Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 35. DECEMBER, 1936. No. 3.

SECTION MEETINGS

ILLINOIS	
Chicago University Medical School	November 25, 1936
IOWA	
State University of Iowa	November 24, 1936
MISSOURI	
St. Louis University Medical School	November 11, 1936
NEW YORK	
New York Academy of Medicine	December 16, 1936
PEIPING	
Peiping Union Medical College	October 28, 1936
SOUTHERN	
Tulane University	December 4, 1936

8974 P*

Cultivation of the Virus of St. Louis Encephalitis.†

R. W. HARRISON AND ELIZABETH MOORE.

From the Oscar Johnson Institute, Washington University School of Medicine, St. Louis.

The virus of St. Louis encephalitis was first cultivated by Syverton and Berry[1] in a living tissue medium composed of minced mouse embryo, rabbit serum and Tyrode's solution. Their results have been confirmed in this laboratory. In pursuing the study further, it has been possible to cultivate this virus in other media[2] and also in the

* P represents a preliminary, C a complete manuscript.

† Conducted under a grant from the Commonwealth Fund of New York.

1 Syverton, J. T., and Berry, G. P., *Science*, 1935, **82**, 596.

2 Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.

developing chick embryo according to the technics adopted for other viruses by Woodruff and Goodpasture[3] and Burnet and Galloway.[4]

Cultures were inoculated with supernatant fluid from 10% suspensions of infected mouse brain. Three strains of the virus were cultivated serially in 4 different media consisting of 0.02 gm. of finely minced mouse or chick embryo suspended in 2.7 cc. of Tyrode's solution or of a mixture of Tyrode's solution and rabbit serum. Transplants were made at 5-day intervals over a period of 4 months. Since no tissue changes attributable to the virus were observed in the cultures, they were tested frequently by intracerebral inoculations of mice. While clinical and pathological findings in mice were identical with those following inoculation with mouse passage virus,[5] culture virus was rarely infective in dilutions higher than 10^{-2}.

Virus inoculated into flasks containing immune serum in place of normal rabbit serum could not be recovered by subculturing in normal serum medium or by inoculation of mice with tissue material washed to remove the antibodies.

After a number of passages *in vitro*, 2 strains of the virus were inoculated into 12- to 16-day chick embryos by placing one or 2 drops of culture upon the chorio-allantoic membrane. Eggs were inoculated in series with tissue from membranes of preceding egg passages and incubated at 38°C. for 2 to 10 days, usually 5 or 6.

The lesion, confined to the chorio-allantoic membrane, appeared first as a cloudy area of proliferation which microscopically involved all 3 layers. In 4 to 7 days the diameter of the lesion reached 1.5 to 2 cm. and its center became necrotic. No pathological changes were found in the body of the embryos although they often died and virus was recovered from brain, liver and spleen in addition to the infected membranes. Mice were infected with tissue from the membranes of each of 7 successive egg cultures of one strain and 10 of the other.

A number of inoculated eggs were allowed to hatch. Most of these embryos died while hatching or within a few hours after emerging from the shell. Sections of the brain of one of these showed extensive perivascular cuffing with mononuclear cells and an occasional small glial nodule.

Of 2 chicks which survived, one was paralyzed in both legs and

---

[3] Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.

[4] Burnet, F. M., and Galloway, I. A., *Brit. J. Exp. Path.*, 1934, **15**, 105.

[5] Smadel, J. E., and Moore, E., *Am. J. Path.*, 1934, **10**, 827.

the other was apparently normal. Brain emulsions from these and from one which died soon after hatching were all lethal for mice inoculated intracerebrally. Virus recovered from the latter was carried through young normal chicks and then through mice by intracerebral inoculation. Clinical signs and pathological changes in the mice were typical of encephalitis. Two of the chicks were paralyzed and brain sections from 3 showed characteristic encephalitic changes. Virus was recovered also from one chick which had no clinical or pathological signs of encephalitis.

Summary. Three strains of St. Louis encephalitis virus have been cultivated in 4 different media containing living tissue and in developing chick embryos. The virus was recovered also from chicks which hatched after inoculation and from young chicks inoculated intracerebrally.

8975 P

Experimental Local Bladder Edema Causing Urine Reflux Into Ureters and Kidneys.

JOHN AUER AND LLOYD D. SEAGER.

From the Department of Pharmacology of St. Louis University School of Medicine, St. Louis.

There are no experimental studies recorded, as far as we are aware, of the rôle that a local transitory edema of the ureteral valve plays in the ureteral reflux of urine. Such a local edema we produced by infiltrating the vesical site of the ureter with 25% magnesium sulphate or with physiological salt solution.

We used 14 guinea pigs, 21 rabbits and 17 dogs, narcotized by morphine, or sodium barbital with or without magnesium sulphate, or ether. In dogs, ether only was used. The bladder and ureters were exposed through a median incision; pressure was recorded through a flanged cannula inserted in the bladder apex, and a water or mercury manometer. India ink was injected into the bladder as indicator. A few times the urethra was ligated. Infiltration of the ureter in its vesical course was done through a fine hypo needle; the amount varied between 0.2 and 2.0 cc.

In 14 guinea pigs (9♂,5♀) 10 showed regurgitation (3♂, 7♀). The pressures ranged between 50 and 280 mm. water (4 to 21 mm. Hg.). In 4 non-pregnant females, spontaneous reflux occurred

into both ureters without infiltration under pressures between 20 and 120 mm. water (2 to 9 mm. Hg.).

In 21 rabbits (12♂, 9♀) 19 showed reflux into one or both ureters. The bladder pressures after infiltration ranged between 30 and 120 mm. water (3– to 9 mm. Hg.). The average control pressure giving no reflux before infiltration was 110 mm. water (9 mm. Hg.).

Spontaneous reflux without infiltration was seen in 2 non-pregnant females, one pregnant female and in one male. In the male and in one non-pregnant female biopsy revealed edematous ureteral orifices and a congested bladder mucosa.

In 17 dogs (8♂, 9♀) ureteral reflux was observed in 13 (6♂, 7♀); no reflux was obtained in 2 males and 2 pregnant females. The bladder pressures necessary for reflux after infiltration varied between 3 and 12 mm. Hg. The average control pressure tested with negative results before infiltration was 8 to 40 mm. Hg. Spontaneous regurgitation without infiltration was seen in 2 females, one of them in early pregnancy.

Biopsy of the bladder in the various species showed usually a well marked edema of the entire vesical ureter section and pouting ureteral orifices. The degree of edema producd by infiltration must not be so great that the lumen of the ureter is occluded. The onset of reflux often occurred during infiltration; the extent varied, but in all series filling of the kidney pelvis with ink was verified by biopsy in numbers of cases. The duration varied; usually after a longer or shorter interval both kidneys and ureters emptied themselves of ink. In some experiments, reflux was produced more than 3 times by successive infiltrations.

Contraction of the bladder did not play an important rôle in partial reflux; in dog for example reflux was produced repeatedly by the hydrostatic pressure of the urine alone (40 mm. water, 3 mm. Hg.). Bladder pressure did play a rôle when ureteral peristalsis was active. Even in these cases a pressure of 6 to 7 mm. Hg. was often sufficient to overcome ureteral peristalsis and drive ink into the kidney pelvis.

Copious secretion of urine may prevent the ascent of ink; under this condition ink only then rose when peristalsis had emptied the ureter. Antiperistaltic waves of contraction from bladder to kidney were never seen.

It seems permissible to assume that edema of the neck of the bladder following operative interferences in that region, or accompanying physiological processes such as menstruation and pregnancy, plays a part in the production of urinary reflux and pyelitis.

8976 C

The Two-Step Oxidation-Reduction of Phthiocol.

EDGAR S. HILL. (Introduced by P. A. Shaffer.)

From the Department of Biochemistry, Washington University School of Medicine, St. Louis.

Having found that semiquinones can be formed from the anthraquinone sulfonates[1] in alkaline solution, it was suspected that phthiocol, a pigment contained in the human tubercle bacillus, isolated by Anderson and Newman[2] and proved by them to be 2-methyl-3-hydroxy-1, 4 naphthoquinone,[3, 4] might behave similarly. Ball[5] studied the electrochemical behavior of phthiocol at pH's ranging from 1.12 to 12.56 and reported no indication of a 2-step oxidation.

Our suspicions were partially confirmed colorimetrically when solutions of phthiocol at pH > 10 were reduced slowly and an intermediate violet color was observed. Solutions of phthiocol were titrated reductively over a pH range from 7.53 to 14.3 (3M-NaOH), and it was found from an analysis of the titration curves that the separation of the 2 steps begins at about pH 9.0 and is more distinct at greater pH's. The separation never becomes so great as to produce a jump of the potential at 50% of the whole titration. The maximum amount of the ratio semiquinone: total dye is about 50% at pH 13.4-14.3. The E'_o curve (midpoint potentials of the system: fully oxidized-fully reduced dye) changes from a slope of .06 to .03 at pH 11.5 (pK_{r_2}) and from .03 to 0.0 in the neighborhood of pH 14.3 (pK_{r_3}). The curves of the midpoint potentials of the systems: semiquinone form-fully reduced form, and totally oxidized form-semiquinone form (E_1 and E_2, respectively as per Elema[6]) cross the E'_o curve at pH 12.9, at a potential of $-.540$ volt.

1 Hill, Edgar S., and Shaffer, Philip A., *Proc. Am. Soc. Biol. Chem.*, 1936, p. li.
2 Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, 1933, **101**, 773.
3 Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, 1933, **103**, 197.
4 Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, 1933, **103**, 405.
5 Ball, E. G., *J. Biol. Chem.*, 1934, **106**, 515.
6 Elema, B., *J. Biol. Chem.*, 1933, **100**, 149.

8977 P

Spectrographic Studies of Lead in Human Blood.*

J. Howard McMillen and Gordon H. Scott.

From the Department of Anatomy, Washington University School of Medicine, St. Louis.

It is of some interest to know the normal distribution of lead in bloods of persons not known to be definitely exposed to lead contamination. We have been able through the courtesy of Dr. J. T. Jean to obtain samples of blood from 89 incoming students. This series, because of probable absence of industrial or occupational hazard, formed a good random sample of the population. This is especially true in view of the fact that the samples were obtained shortly after the vacation period. As might be expected our series contained a larger number of St. Louis inhabitants than one would ordinarily select for such a study. The group being medical students was predominantly male. Only 6 females are represented in the series.

Blood was drawn into pyrex test tubes which had been cleaned with nitric acid. After the blood had clotted small pieces of the clot were placed on the ends of carbon electrodes and dried by moderate heat. The residue was then burned in the flame of an intermittent arc. Ordinarily the samples ignited without difficulty. Since 100 flashes consumed nearly all the material on the electrode it was necessary to prepare 3 separate samples. With the flashes occurring at 80 per minute, 300 flashes gave the proper exposure. A Bausch and Lomb medium quartz spectrograph with a slit width of 0.015 mm. was used.

For the quantitative estimations the intensity of the 2833.07 Pb line was compared with the 2831.56 Fe line (Scott and McMillen[1]). The relative densities of the iron and lead lines were interpreted in terms of absolute values by a series of blank runs of rabbit blood to which known amounts of lead had been added. Our measurements of line density were made with a recording microphotometer.

Of the 89 members of the series 45 showed definite traces of lead in quantities of 1×10^{-8} gm. or more per cc. of whole blood. There

* Aided by grants from the Josiah Macy, Jr., Foundation, the National Research Council, the C. M. Warren Fund of the American Academy of Arts and Sciences, and the Rockefeller Foundation to Washington University for research in science.

1 Scott, Gordon H., and McMillen, J. H., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 287.

were 11 with from 1 to 3×10^{-8} gm. of lead; 20 from 4 to 6; 5 from 7 to 9; 4 from 1 to 1.2×10^{-7}; 3 from 1.3 to 1.5; 2 from 1.6 to 1.8×10^{-7}. It will be noted that the greatest number of the cases showing lead fell around 6×10^{-8} gm. None of the members of the series show an amount of lead greater than 1.9×10^{-7}. By way of comparison it may be pointed out that a definite case of plumbism contained 3.2×10^{-6} gm. of lead per cc. of blood. This case falls within the limits of the pathological range of 2×10^{-6} to 1×10^{-5} gm. of lead per cc. of blood set by Blumberg and Scott.[2]

8978 P

Initiation and Maintenance of Lactation in Hypophysectomized Guinea Pigs.*

E. T. GOMEZ AND C. W. TURNER.

From the Department of Dairy Husbandry, Missouri Agricultural Experiment Station.

The somewhat purified preparation of the lactogenic hormone (galactin), unlike crude pituitary extracts, failed to initiate or to maintain lactation in hypophysectomized guinea pigs.[1, 2] These observations were not taken to indicate the inadequacy of the lactogenic hormone in stimulating secretory activity of the mammary epithelium but rather as a result of the general physiological disturbance in the hypophysectomized animal which invariably reduces to a low level the available precursors of milk in the blood.

There is an increasing amount of evidence that the pituitary secretes hormones which directly or indirectly through their action upon other endocrine glands, regulate the composition of the blood and therefore play an important indirect rôle in milk secretion. Graham[3] and Gaunt and Tobin[4] have indicated the importance of the thyroid and adrenal cortex in relation to milk secretion.

Nelson and Gaunt[2] reported that the administration of Swingle-

2 Blumberg, H., and Scott, T. F. M., *Johns Hopkins Hosp. Bull.*, 1935, **56**, 32.

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 483.

1 Gomez, E. T., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 404.

2 Nelson, W. O., and Gaunt, R., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 671.

3 Graham, W. R., Jr., *J. Nutrition*, 1934, **7**, 407.

4 Gaunt, R., and Tobin, C. E., *Anat. Rec.* (Supp.), 1936, **64**, 18.

Pfiffner's adrenal cortical extract in hypophysectomized guinea pigs failed to initiate lactation or prevent its cessation. However, the lactogenic hormone plus adrenal cortical hormone initiated lactation in 12 hypophysectomized guinea pigs, and by continued treatment milk secretion was maintained for about 5 days.

The present paper presents results which confirm and extend the above observations. The spontaneous hypoglycemic coma which develops in hypophysectomized guinea pigs was successfully controlled to a great extent by the daily administration of 1 cc. of 20% glucose solution per 100 gm. body weight. In the experiments to be described it should be recognized that this solution played an important rôle in maintaining the level of blood glucose which is an important precursor of milk.

Initiation of lactation. Six sexually mature male and 4 involuted multiparous female guinea pigs† which were prepared for lactation by the injection of theelin (in oil)‡ daily for 20 days were hypophysectomized 24 hours after the last injection. Immediately after hypophysectomy, one of the males was given 0.2 dog unit of adrenal cortical hormone (eschatin)‡ and another 5 mg. of lactogenic hormone (galactin). The rest were injected with 5 mg. of galactin and eschatin in amount ranging from 0.2 to 0.4 dog unit administered simultaneously by subcutaneous injection daily for a period of 5 to 7 days, when the animals were sacrificed.

The animals receiving galactin or eschatin alone showed no evidence of lactation during a period of 7 days. In all instances, however, lactation was induced within 2 to 3 days after the first injection in animals receiving galactin and eschatin; and by continued treatment milk secretion was maintained throughout the observation period of 5 to 7 days. With the exception of 2 animals which showed microscopic evidence of anterior pituitary fragments in the sella turcica, all the animals were found to be completely hypophysectomized.

Maintenance of Lactation. Ten lactating guinea pigs, hypophysectomized 2 to 3 days after parturition, were immediately injected with 3 to 5 mg. of galactin and 0.2 to 0.4 dog unit of eschatin simultaneously once daily for periods ranging from 5 to 20 days. Gross daily observations of these animals before the young were allowed to nurse after being kept away over night revealed abundant milk secretion in the mammary glands. However, the amount of

† A considerable number of animals which succumbed to the treatment too soon to allow for the necessary observations are omitted from this report.

‡ These hormones were kindly and generously supplied by Dr. Oliver Kamm of Parke, Davis and Company.

milk secreted declined gradually so that by the 10th to 20th day of injection only a few drops of milk could be expressed from the nipples. In spite of continued milk secretion, there appeared to be a gradual involution of the mammary gland. In 2 similar cases in which galactin alone was injected, milk secretion stopped in 3 days after the first injection. Lactation was reinitiated in these animals within 3 days with 5 mg. of galactin and 0.2 dog unit of eschatin, injected immediately after the cessation of lactation and maintained throughout the observation period of 10 days.

Summary. The lactogenic hormone (galactin) in conjunction with the adrenal cortical hormone (eschatin) and glucose was capable of initiating or reinitiating lactation in hypophysectomized guinea pigs with properly conditioned mammary glands. Though simultaneous injections of these 2 hormones and glucose support the maintenance of lactation in hypophysectomized lactating guinea pigs, the involution of the mammary glands was only slightly, if at all, prevented.

8979 P

Influence of Suckling upon Galactin Content of the Rat Pituitary.*

R. P. REECE AND C. W. TURNER.

From the Department of Dairy Husbandry, Missouri Agricultural Experiment Station.

We have reported[1] that the pituitary glands from 48 hour postpartum rats contained, on the average, 7.72 bird units of galactin per gland. In this experiment the young were with the mother continually up until the time when she was sacrificed.

In the light of Selye's[2] work upon the nervous control of lactation it became exceedingly interesting to study the influence of suckling upon the galactin content of the rat pituitary. The litters of 10 rats were removed 36 hours after parturition and then returned to their mothers 12 hours later, without receiving any nourishment from other lactating rats during this 12-hour period. Frequent observations were then made to see if the young were nursing.

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 485.

1 Reece, R. P., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED. (in press).

2 Selye, Hans, *Am. J. Physiol.*, 1934, **107**, 535.

Immediately after a nursing period of 3 hours the mother rats were sacrificed, their pituitaries removed, weighed and assayed. For controls the litters of 10 rats were removed 36 hours after parturition and the mother rats sacrificed 15 hours later. The pituitaries were assayed by injecting the macerated tissue, suspended in distilled water, intradermally over the crop gland of common pigeons. The pituitary glands were injected into a sufficient number of pigeons so as to obtain a minimal response of the crop gland.

TABLE I.

Rat No.	Body Wt. gm.	Total Pituitary Wt., mg.	Bird Units per Pituitary Gland	Bird Units per 1 mg. Pituitary Tissue	Bird Units per 100 gm. Body Wt.
36*	266	12.5	5.50	.44	2.07
38*	232	9.8	2.37	.24	1.02
41*	225	9.4	2.67	.28	1.19
24*	213	10.4	1.50	.14	0.70
21*	206	10.6	5.50	.52	2.67
18*	191	8.4	2.00	.24	1.05
42*	190	9.9	3.50	.35	1.84
40*	187	9.7	2.50	.26	1.34
28*	183	9.2	2.25	.24	1.23
39*	175	9.2	2.87	.31	1.64
Aver.	206	9.9	3.06	.30	1.48
26†	230	13.7	10.50	0.77	4.57
46†	227	10.4	10.25	0.99	4.52
23†	217	11.5	6.75	0.59	3.11
49†	207	10.5	9.25	0.88	4.47
50†	191	9.2	8.50	0.92	4.45
43†	176	8.1	9.25	1.02	5.26
44†	169	7.4	8.67	1.17	5.13
47†	167	7.3	9.00	1.23	5.39
45†	162	7.2	11.37	1.58	7.02
48†	156	7.0	8.50	1.21	5.45
Aver.	190	9.2	9.20	1.04	4.94

* Experimental animals (suckled).
† Control animals (non-suckled).

From the results presented in Table I, it appears that the cessation of nursing and the accumulation of milk in the glands permits an augmentation of the galactin content in the pituitary gland (9.20 bird units of galactin as compared with 7.72 units). Following the stimulus of suckling and the removal of milk from the mammary glands the galactin appears to be discharged to a remarkable extent, the pituitary glands from the non-suckled rats containing 3 times as much galactin as the pituitaries from suckled rats.

Conclusion. The stimulus of suckling or the removal of milk from the mammary glands markedly decreases the galactin content of the rat pituitary gland.

8980 P

Further Studies on Action of Physostigmine on Autonomic Ganglia.

THEODORE KOPPANYI, ROBERT P. HERWICK AND CHARLES R. LINEGAR.

From the Department of Pharmacology and Materia Medica, Georgetown University School of Medicine, Washington, D. C.

A number of authors[1-6] have described the vasopressor effect of large doses of normally vasodilator choline derivatives in atropinized animals, including the "parasympathetic substance" acetylcholine. The findings of Hunt[7] and Broun and Beaune[8] on these subjects will be discussed in a detailed paper.

All drugs used in these experiments were given intravenously and the doses expressed in milligrams per kg. In 6 cats, 4 rabbits and 10 dogs anesthetized with barbiturates, it was found that acetylcholine in doses of 0.05 to 0.1 mg. did not produce vasopressor effects following the administration of 2 to 5 mg. of atropine sulphate. If the administration of acetylcholine was preceded by 0.3 to 2 mg. of physostigmine salicylate in atropinized animals, acetylcholine produced sharp, epinephrine-like rises in the blood pressure of 50 to 100 mm. of Hg. in cats and dogs, and lesser rises in rabbits. These pressor effects with acetylcholine may be elicited many times over a period of 6 hours in atropinized animals following a single dose of 0.5 to 1.0 mg. of physostigmine salicylate.

This action of physostigmine on the responses of small doses of acetylcholine in atropinized animals may be ascribed to a ganglionic action of this drug in facilitating the cholinergic effect of acetylcholine through the sympathetic synapses.

The injection of 3 mg. of cocaine hydrochloride enhanced the vasopressor effects of acetylcholine under the above conditions, but could not elicit vasopressor responses from small doses of acetylcholine in a physostigmine-like manner.

The injections of 0.1 to 1.0 mg. of pilocarpine produced vasodilator effects which were completely antagonized by atropine. Fol-

1 Schmiedeberg, O., and Koppe, R., *Das Muscarin*, Leipsic, 1869.
2 Jordan, S. N., *Arch. Exp. Path. and Pharm.*, 1878, **8**, 25.
3 Boehm, R., *Arch. Exp. Path. and Pharm.*, 1885, **19**, 99.
4 Mott, F. W., and Halliburton, W. D., *Proc. Roy. Soc.*, 1899, **141**, 211.
5 Hunt, R., and Taveau, R., *U. S. Hyg. Lab. Bull.*, 1911, **73**, 17.
6 Dale, H. H., *J. Pharm. and Exp. Therap.*, 1914, **6**, 147.
7 Hunt, R., *Am. J. Physiol.*, 1918, **45**, 231.
8 Broun, D., and Beaune, A., *C. R. Soc. Biol.*, 1936, **121**, 1589.

lowing atropinization, pilocarpine even in doses of 10 to 50 mg. produced no typical effects in the presence of physostigmine. Pilocarpine, unlike physostigmine, did not elicit pressor effects from small doses of acetylcholine in atropinized animals.

The mechanism of pilocarpine action is fundamentally different from that of physostigmine.

8981 P

Further Studies on Physostigmine-Nicotine Antagonism.

CHARLES R. LINEGAR, ROBERT P. HERWICK AND THEODORE KOPPANYI.

From the Department of Pharmacology and Materia Medica, Georgetown University School of Medicine, Washington, D. C.

It has been reported by several authors[1-4] that large doses of nicotine (30 to 60 mg. per kg.) prevented the pressor effects of acetylcholine (1 to 5 mg. per kg.) in atropinized animals.

The drugs used in these experiments were all given intravenously and the doses expressed in milligrams per kg. In 6 dogs and 6 cats anesthetized with barbiturates and treated with 2 mg. atropine sulphate and 1 mg. of physostigmine salicylate, pressor effects were elicited in every case following the injection of 0.05 to 0.1 mg. of acetylcholine. The administration of paralytic doses of nicotine salicylate (3 to 6 mg.) in addition to the above drugs changed the effects of acetylcholine injections to uniformly vasodilator responses. Nicotine thus caused a reversal of the hemodynamic action of acetylcholine under the above conditions. In 5 cats and 5 dogs, it was further found that paralytic doses of nicotine are not necessary to obtain this reversal. Doses of nicotine from 0.25 to 2 mg. always diminished and often reversed the hemodynamic effect of acetylcholine. Further doses of physostigmine salicylate again reversed the acetylcholine vasodepressor effects into pressor responses. The administration of nicotine again changed these pressor responses of acetylcholine into depressor effects. This mutual antagonism between physostigmine and nicotine in reversing the acetylcholine blood pressure effects can be demonstrated several times in the same animal.

[1] Dale, H. H., *J. Pharm. and Exp. Therap.*, 1914, **6**, 147.

[2] Hunt, R., *Am. J. Physiol.*, 1918, **45**, 231.

[3] Fuehner, H., *Biochem. Z.*, 1916, **76**, 232.

[4] Feldberg, W., and Minz, B., *Arch. Exp. Path. and Pharm.*, 1931, **163**, 66.

8982 C

Potentials from the Isolated Forebrain and Potential Summation in the Isolated Brain of Catfish.

P. Sheridan Shurrager.* (Introduced by Fred W. Tanner.)

From the Physiology Laboratory, Clark University.

Electrical responses of smooth wave form from the midbrain and vagal lobes of the completely isolated brain of the goldfish (*Carassius auratus* L.) have been described by Adrian and Buytendijk.[1] These waves were apparently periodic states of negativity in the vagal lobes relative to an earthed electrode, and occur at a frequency roughly corresponding to the normal opercular beat. Adrian and Matthews,[2] working on electrical waves from the anesthetized cortex of the rabbit, varied the distance between balanced electrodes and found that the long, smooth waves require large areas for their propagation. They concluded, as I have found using a single grid electrode, that the smooth wave contours are summed potentials of relatively short duration. For recent reviews of the extensive literature on electrical waves of central origin see Kornmuller,[3] Jasper,[4] Prosser.[5] It is generally conceded that the spontaneous discharges in the central nervous system have their origins in the nerve cells (Adrian and Buytendijk[1] Bartley and Bishop[6] Adrian and Matthews[2]; and the reviews just cited). Hoagland[7] has presented evidence indicating that the alpha waves from the occipital cortex of man arise as "relaxation oscillations" at frequencies directly proportional to the rate of the respiration of the cortical tissue.

After cutting the cord posterior to the medulla, brains of catfish, *Ameriurus nebulosus* (LeSueur), were removed and placed on a glass plate moistened with isotonic NaCl solution (0.089 N). This solution bathing the tissues prevents drying and sustains the activity. Records obtained from brains bathed with catfish blood serum did not differ from those obtained when isotonic saline was used. I am informed that according to Gerard (Symposium discussion), who

* At present on the Research Staff of The Physiological Psychology Laboratory, University of Illinois.

1 Adrian and Buytendijk, *J. Physiol.*, 1931, **71**, 121.

2 Adrian and Matthews, *J. Physiol.*, 1934, **81**, 440.

3 Kornmuller, v. A. E., *Biol. Rev.*, 1935, **10**, 383.

4 Jasper H., *J. Gen. Psychol.*, 1936, **14**, 98.

5 Prosser, C. L., *Cold Spring Harbor Symposia*, 1936, Vol IV (in press).

6 Bartley and Bishop, *Am. J. Physiol.*, 1933, **103**, 173.

7 Hoagland, H., *Am. J. Physiol.*, 1936, **116**, 604.

has obtained records from frog brains, isotonic NaCl bathing the brain gives essentially the same sort of records obtained when the brain is bathed with Ringer's solution. The dissections were performed with as little traumatic effect as possible. When care is taken to preserve the respiratory movements and blood supply until the operation is almost complete, the most stable and enduring preparations are obtained. Injuries to nuclei generally result in total lack of spontaneous activity. About half of the brains examined were found to be inactive. Oscillations were obtained from some 30 brains.

Continuous records were made on paper tape with a Garceau ink-writing undulator and amplifier system. The undulator records (Figs. 1 and 2) were then photostated or traced. The time constant of the amplifier was such that a sustained pulse of 10 to 100 μv. fell to zero in approximately 200 milliseconds. Some damping of the waves due to properties of the undulator was observed. However, comparisons of the undulator records with the direct coupled amplifier records which Adrian and Buytendijk[1] obtained with a Matthews oscillograph show much similarity.

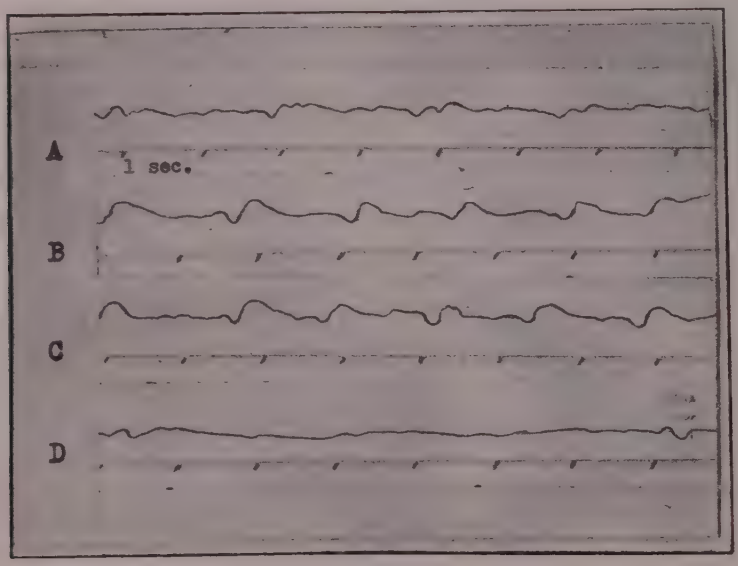


FIG. 1.

Spontaneous discharges from the intact and isolated cerebral component of the catfish forebrain. A, period of relatively asynchronous activity; B, the typical smooth wave from the intact forebrain; C, the isolated response; D, decreased potentials after isolation of 3.5 minutes.

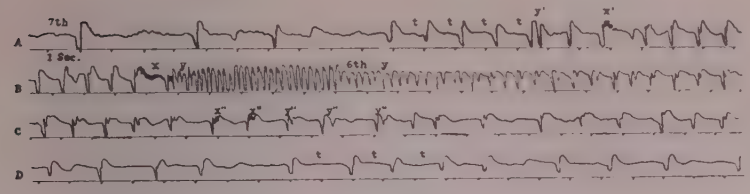


FIG. 2.

A continuous record (A-B-C through D) of electrical oscillations from the cerebral lobe region of the isolated catfish brain. t, the typical discharge from the cerebral lobe; y′ and x′ show regions of instability and precursors of change; x, summation within the typical wave form; y, period of reorganization of the summed potentials; x″ and y″, regions of greatest summation and reorganization.

Cotton wick electrodes were attached to chlorided silver loops and saturated with isotonic saline. The grid wick electrode was placed lightly on the surface of the tissue; the placement of the earthed electrode had no effect upon the wave form, thus indicating that all the electrical changes recorded apparently take place under the single grid electrode. When the waves were in progress, crushing the tissue, or the application of formalin abolished them.

Frequently when electrical responses had ceased in the medulla and midbrain, it was possible to secure rhythmical responses from either the distal (olfactory) component, or the mesial (primitive cerebrum) component of the forebrain. Records from these components showed considerable differences in form. The cerebral component gave waves resembling the smooth midbrain waves described by Adrian and Buytendijk,[1] while the olfactory component produced waves similar in form to the discharges from the medulla recorded by these workers.

Isolation of the forebrain, accomplished by severing with a sharp scalpel, often does not stop the rhythmical responses, which may continue for 5 or 6 minutes (Fig. 1-C) before decreasing in amplitude and frequency (Fig. 1-D) and finally disappearing entirely. The disappearance of these waves is probably similar to the gradual disappearance of the spontaneous potentials which occurs in the visual cortex of the rabbit after cutting and ligation as observed by Bartley and Bishop.[6]

It is interesting to note that when there is a gradual decline in the amplitude and frequency of the major discharges, there often appears between these discharges a series of minor deflections (Fig. 1-D and Fig. 2-A) which are comparable to other rhythmical discharges sometimes observed in the series of waves.

Because the condenser system employed maintains a D.C. pulse for a maximum of 200 milliseconds, and the discharges which sustained the slow waves were frequently more than 1000 milliseconds

in duration, it was evident that these slow waves were summed potentials.

The smooth, rhythmic waves infrequently showed disintegration and reconstruction periods. Figure 2 is illustrative of these transitions, and suggests that the waves are sustained in their various forms when these summing potentials are repeatedly synchronized and combined in their various complex forms according to their own shifting phase relations.

Summary. 1. Rhythmic electrical potentials of from 10 to 180 μv. have been recorded from the isolated olfactory and cerebral lobes of the forebrain of catfish. The "spontaneous" potentials from the 2 different regions differ in form. 2. The smooth wave contours of the central nervous system are apparently complex summed potentials of many smaller oscillations.

8983 C

Higher Resistance of Rats Fed Casein Than Those Fed Vegetable Proteins.

ELIZABETH CHANT ROBERTSON AND MARTHA ELIZABETH DOYLE.
(Introduced by F. F. Tisdall.)

From the Research Laboratories of the Department of Pediatrics, University of Toronto, and the Hospital for Sick Children, Toronto, under the direction of Dr. Alan Brown.

Although it has been well established that rats fed a vegetarian diet are inferior to those fed an omnivorous diet both as regards body weight and lactation,[1] little is known as to their relative resistance to infection. Chen and Li[2] found that when rats fed a vegetarian diet made up of wheat, millet, soy bean and peas, and others fed an omnivorous diet of wheat, milk powder and fresh vegetables were infected with hog cholera bacilli I.P., 84% of the former and 42% of the latter died. When pneumococci (Type I) were injected subcutaneously, more of the omnivorous (77%) than of the vegetarians (56%) died, but according to these authors this difference is not great enough to be significant statistically. We thought it of interest to test the resistance of rats fed diets varying only in the types of protein which they contained. The diets were constituted as follows:

1 Editorial *J. Am. Med. Assn.*, 1935, **105**, 438.

2 Chen, T. T., and Li, C. P., *Chinese J. Physiol.*, 1930, **4**, 59.

	Gluten Diet %	Soy Bean Diet %	Casein Diet %
Wheat Gluten	17	—	—
Soy bean flour	—	21	—
Casein	—	—	17
Cornstarch	56.5	53.5	56.5
Crisco	10	9	10
Cod liver oil	2	2	2
Wheat germ	10	10	10
F.R.L.[3] Salt Mixture	4.5	4.5	4.5

All 3 diets contain 13.6 to 13.9% of protein and as the fat content in the soy bean flour was 4% instead of 0.7 to 0.3% as in the other proteins, the fat in the former diet was reduced a little.

Litters of 4-weeks-old rats were divided into lots of 3 and fed the above diets for 4 weeks, when their average weights were, gluten diet 96, soy bean diet 86, and casein diet 123 gm. respectively. We had planned to feed broth cultures of *B. enteritidis*, but the strain had lost its feeding pathogenicity and would not kill when given by mouth. The rats were therefore given intraperitoneal injections of an 18 hours' broth culture of *S. enteritidis*. As shown in Table I, quite a large number of the rats died, and with 3 exceptions all of the deaths occurred within 2 weeks after infection. The dead rats all showed diarrhea, bloody nasal discharge and blood about the eyes, and the organism was always recovered from the heart's blood and identified by agglutination.

In the first experiment, all of the rats were given the same dose, but in the second, they were given doses proportional to their weights, *i. e.*, .007 cc. of a 1 in 50 dilution of the broth culture per gram of body weight (Table I).

TABLE I.

	Dose cc.	Gluten Diet No. of rats	Gluten Diet No. dead	Gluten Diet % alive	Soy Bean Diet No. of rats	Soy Bean Diet No. dead	Soy Bean Diet % alive	Casein Diet No. of rats	Casein Diet No. dead	Casein Diet % alive
Exp. 1	.0066	19	13	31	18	11	39	22	3	86
" 2	prop. to weight	22	17	23	20	17	15	20	7	65

In both the experiments there was no difference between the survival rate of the rats fed the gluten diet and of those fed the soy bean ration. There was, however, a definite difference between the high resistance of the casein-fed rats and the low resistance of those fed either the gluten or soy bean diet. In Exp. 1, the chances are 300 to 1 that the difference is statistically significant[4]; and in Exp. 2, they are 800 to 1.

[3] Hawk, P. B., and Oser, B. L., *Science*, 1931, **74**, 369.

[4] Topley, W. W. C., *An Outline of Immunity*, Arnold, London, 1933, p. 18.

Conclusion. Rats fed a diet containing casein have a considerably higher resistance to enteritidis infections (I.P.) than controls fed either wheat gluten or soy bean flour.

8984 C

A Biochemical Effect of Ether on the Gut.

GEORGE A. EMERSON.

From the Department of Pharmacology, West Virginia University School of Medicine, Morgantown.

Physiological techniques have demonstrated[1] a marked inhibition of the gut during ether anesthesia and for a short time during recovery. In other cases of reduced motility of the gut, as in atonic constipation, complementary regular changes in putrefaction have been noted. Difficulty has been encountered in showing any such regular changes in the excretion of putrefactive bodies in the urine of anesthetized subjects.[2] It was felt that more distinct changes could be noted by examining the fecal material directly.

Bergeim[3] introduced a technique for determining the putrescibility of proteins *in vivo*, which depends essentially on observation of the stronger reduction processes occurring in the gut contents. This test may equally well be used to note changes in putrefaction of a single protein due to physiological factors such as motility of the gut. When so used, it is not subject to certain criticisms[4] of the original method, since the diet is constant. Two of Bergeim's[3] protein diets* were used in the following experiments: casein, a protein of low putrescibility, and egg albumin, which is more highly putrescible. Because of the extended period the rats were fed these diets, 2% of Wesson's[5] salt mixture were added.

Reduction processes in the gut associated with putrefaction[3] may be estimated by noting the per cent reduction of ingested ferric oxide incorporated in the Bergeim diets. Feces containing the Fe^{++}-Fe^{+++} mixture are heated at 100°C. for 10 minutes with dilute HCl, and a

1 Miller, G. H., *J. Pharm. Exp. Therap.*, 1926, **27**, 41.

2 Killian, H., *Narkose zu operativen Zwecken*, Berlin, 1934.

3 Bergeim, O., *J. Biol. Chem.*, 1924, **62**, 45, 49.

4 Hoelzel, F., *J. Biol. Chem.*, 1929, **83**, 331.

* White dextrin, 800 gm.; casein or egg albumin, 200 gm.; granulated agar, 10 gm.; ferric oxide, finely powdered, 10 gm.; salt mixture, 20 gm.

5 Wesson, L. G., *Science*, 1932, **75**, 339.

TABLE I.

Ferric Reduction in the Gut of Rats fed a Dextrin-Casein Diet. Results Expressed in % Fe^{++} of Total Iron.

Group	No. of Rats	Treatment	Ferric Reduction, Days of Diet							
			1	2	3	4	5	6	7	11
A	10	Ether, 2.5 mM/L. for one hour on 4th day	61±2	44±2	29±2	*28±2*	21±4	17±2	13±3	14±2
B	10	Similar treatment, but repeated 5th and 6th days	70±4	44±5	31±2	*27±3*	*34±3*	*27±2*	15±2	17±2
C	10	Epinephrine HCl, divided doses, 4th to 6th days, as in text	52±5	39±2	31±4	*21±3*	*23±3*	*16±2*	18±2	*26±3
D	10	Untreated controls	60±2	60±4	27±4	24±2	25±3	16±1	14±2	17±3
Aver.			61	47	30	—	—	—	15	16

*Large sloughs at site of injection in 2 rats; necrotic material eaten by other rats.

TABLE II.

Ferric Reduction in the Gut of Rats Fed a Dextrin-Albumin Diet. Results Expressed in % Fe^{++} of Total Iron.

Group	No. of Rats	Treatment	Ferric Reduction, Days of Diet								
			1	2	3	4	5	6	7	8	10	15†
A	10	Ether, 2.5 mM/L. for one hour, 4th to 7th days	46±3	55±2	41±3	*43±2*	*55±2**	*54±4*	*64±3*	33±4	35±2	17±3
B	9	Similar treatment, 4th to 6th days	49±4	37±2	43±4	*48±3*	*59±3*	*55±4*	37±4	36±4	34±2	16±1
C	10	Ether, middle of 4th day	46±5	42±4	40±4	*46±2*	48±3	37±4	37±3	37±2	35±4	19±3
D	9	Untreated controls	56±4	39±2	39±2	40±2	50±3*	41±4	38±2	37±5	34±2	19±4
Aver.			49	43	41	—	—	—	—	36	35	18

*One rat died in each of groups A and D; transient high reduction values resulted from other rats of these groups eating the carcasses.

†Fasted on 11th day and fed Dextrin-Casein diet 12th to 15th days.

colorimetric comparison of the Fe^{++} content of 2 aliquots of the filtrate from this mixture is made after treating one aliquot with $KMnO_4$. The per cent reduction may thus be obtained directly, without the necessity of determining actual total Fe present. Modifications of Bergeim's[8] technique which were found to increase the accuracy of the method were: use of boiled distilled water to make up the dilute HCl; 10 minutes heating; and the use of mineral oil to exclude atmospheric oxygen during the heating.

Tables I and II illustrate changes in putrefaction brought about by repeated ether administration. Four groups of 9 or 10 rats each were used for each diet. Feces were collected by digital pressure on the rectum, 24 hours after beginning feeding of the diets and in the same way at the same time each day thereafter. Feces were immediately analyzed after collection, as above. When the groups had approached a stable point as noted by lowering of the standard deviation of the mean and agreement of results in the 4 groups, at the beginning of the 4th day, treatment was instituted in 3 of the groups while the 4th was kept as untreated controls. Of the groups receiving casein, the first was anesthetized with 2.5 mM/L. of ether in oxygen for one hour at the beginning of the 4th day, the 2nd was similarly treated on the 4th to 6th days, while the 3rd group was treated with 3 doses of 0.2 mg./kg. of epinephrine HCl given subcutaneously at 4-hour intervals on the 4th and 5th days respectively, and 4 similar doses given on the 6th day. This follows Cori's[9] suggestion for "continuous" epinephrine administration. Of the groups receiving egg albumin, the first was treated similarly with ether at the beginning of the 4th to 7th days, the 2nd on the 4th to the 6th days and the 3rd at the middle of the 4th day.

Table III represents changes in putrefaction immediately after treatment. Two groups were used of rats previously fed the casein diet until their feces showed very low reduction. One group was observed without treatment for a day, and then given a single subcutaneous dose of 0.5 mg./kg. of epinephrine HCl. The other group was treated with ether for one hour on the first day and observed without treatment on the following day. It was found difficult to collect feces from all of the treated animals at each point, but a sufficient number were obtained to yield significant results. The average number of determinations at each point excepting the 2 noted in the table as single observations was 8.6 for the control groups and 7.7 for the experimental groups.

[9] Cori, C. F., *Physiol. Rev.*, 1931, **11**, 143.

TABLE III.

Course of Intestinal Reduction after Single Drastic Treatments. Groups of Ten Rats Fed Bergeim's Dextrin-Casein Diet.* Results Expressed in % Fe^{++} of Total Iron.

Group	Treatment	Before Treatment	Time in hours after start of treatment: 1	2	3	4	5	6	8	10	12	18	24
A	Controls, untreated, 1st day	16±1	16±1	—	16±2	—	16±1	—	16±1	—	16±1	—	16±1
	Single dose 0.5 mg./Kg. Epinephrine HCl 2nd day	16±1	16±2	18±3	19±3	18±2	(14)	—	17±2	21±3	21±2	28±2	31±4
B	Ether, 2.5 mM/L. for one hour. 1st day	16±1	(21)	17±2	19±1	20±3	21±2	23±3	21±2	22±2	22±3	20±3	17±2
	Controls, untreated, 2nd day	17±2	—	16±2	—	17±2	—	—	—	16±2	—	—	17±1

*Maintained on casein diet 6 days before starting treatment.

— blank spaces indicate collection of feces without analysis; control of influence of manipulation.

( ) parentheses indicate result of a single observation only.

The results as tabulated may be compared only with values for the control groups maintained on the same diet, as it may be seen readily that the type of protein fed has far greater influence on reduction of ferric iron than does stasis. It may be noted that the usual laboratory diet for rats is highly putrescible, as shown by initial values in Tables I and II.

If the results are examined in this way, it appears that a significant variation from the controls occurs on the 3rd day of repeated ether administration, in animals fed the casein diet. Repeated treatment with epinephrine produces little effect, possibly because the gut has sufficient time to recover activity before the fecal samples are taken. With animals on the albumin diet, the standard deviation of the mean is about the same as with casein-fed rats, but the reduction is greater, so that a significant variation appears on the 2nd day of repeated etherization. In the single acute experiments of Table III, it appears that the peak of the effect of ether is passed by the 12th hour, so that greater effects would have appeared in Tables I and II if 12-hour samples had been taken in place of those at 24 hours. The marked, continued effect of 0.5 mg./kg. of epinephrine HCl is in contrast to the effect of 0.2 mg./kg., and the latency of the effect is further indication of its extent, since this latency is probably a reflection of the slower passage of feces. The closer similarity between the effects of ether and 0.5 mg./kg. of epinephrine than 0.2 mg./kg. occurs elsewhere,[7] also.

Since 24-hour urine specimens of patients anesthetized with ether or other anesthetics showed no regular changes in concentration of indican, urobilinogen, ethereal sulphates or free and conjugated phenols during 2-3 days post-anesthetically, no attempt was made to collect sufficient cases to give statistically significant results. The findings agree with those in the literature,[2] and may be explained through pre-anesthetic medication, time of anesthesia and the nature of the diet before and after anesthesia.

Summary. Ether acts to increase reduction processes in the gut of rats, and gives rise to significant changes in these processes if the anesthesia is repeated on successive days. Epinephrine HCl showed no such effect on fecal samples taken 24 hours after beginning treatment with 0.2 mg./kg. given subcutaneously at 4-hour intervals for 8 or 12 hours, but a single dose of 0.5 mg./kg. resulted in a marked increase of reduction in fecal samples taken from 10-24 hours later. Reduction processes in the rat gut after surgical anesthesia with ether for one hour reach a peak with the fecal sample

[7] Emerson, G. A., *J. Pharm. Exp. Therap.*, 1935, **55**, 90.

taken 6 hours after induction of anesthesia, and show some augmentation over those of untreated controls up to the 18th hour after anesthesia. These definite and regular biochemical corollaries of the functional disturbance in ether anesthesia are not also found uniformly reflected in the urinary excretion of putrefactive bodies by surgical patients.

8985 P

Response of Chick Testes and Ovaries to Rat Pituitary Implants.*

R. L. KROC AND W. R. BRENEMAN. (Introduced by F. L. Hisaw.)

From the Waterman Institute, Indiana University.

Implants of rat pituitaries were made into 98 male and 40 female chicks as part of a program of study of the pituitary-gonad inter-relationship. The results were unexpected, and we believe of general interest because of possible bearing on the problem of species specificity of hormone action. The bird affords an excellent test for experiments involving pituitary hormones of animals in different classes because: (1) of the marked response of the immature testis to the "follicle stimulating" hormone (F.S.H.),[1] and, (2) since it was reported by one of us,[2] that the testes of chicks given the pituitary luteinizing principle differed histologically from those of chicks which received F.S.H.

Young adult rats weighing 180-225 gm. were used as donors and those castrated were operated upon 9 weeks previous to the time of implantation. Weights of pituitaries used corresponded closely to those tabulated for Wistar rats of similar weight.[3] Single pituitaries were implanted subcutaneously into the chicks on the 5th and 7th days after hatching and the birds were killed on the 9th day. Control experiments with implants into immature female rats demonstrated the same quantitative difference in ovarian weights which has been reported by other workers.[4] A summary of the results in the chick appears in Table I.

*Contribution No. 74 from the Waterman Institute, and No. 262 from the Zoology Department of Indiana University.

1 Riddle, O., and Bates, Robert W., *Endocrinology*, 1933, **17**, 689.

2 Breneman, W. R., *Anat. Rec.*, 1936, **64**, 211.

3 Donaldson, H. H., 1924, *The Rat*, Philadelphia, 256.

4 Evans, H. M., and Simpson, M. E., *Am. J. Physiol.*, 1929, **89**, 371.

These data indicate quite definitely that the increased weight observed for the testes and ovaries did not demonstrate the striking differential weight increment found when immature female rats were recipients of implants. There was moreover, no statistically significant difference between the averages of the various male or female groups. In contrast to the rat, the heaviest gonads occurred in cockerels which were given pituitary implants from normal females. Four pairs of testes weighed respectively 57.4 mg., 46.2 mg., 46 mg., and 45 mg. (exceeding the maximum for all other series, namely, 39 mg.). This observation necessitates further check to determine if the stage of the reproductive cycle influenced the physiological action of the female pituitaries in these experiments.

TABLE I.
Weight Response of Chick Gonads to Subcutaneous Pituitary Implants.
Gonad weights are in mg. for the 2 testes or for the left ovary.

Implant	No.	Range	Aver.
		Male Chicks.	
None	23	6.0-21.5	11.12±0.96
♀	38	8.1-57.4	21.28±1.62
♀*	17	14.0-31.5	21.51±1.24
♂	24	14.0-34.8	20.48±0.87
♂*	19	14.5-39.0	22.16±1.38
		Female Chicks.	
None	15	7.0-16.0	11.35±0.68
♀	11	11.5-21.5	16.69±0.93
♀*	10	10.0-20.0	14.99±0.94
♂	8	10.5-20.5	16.66±1.13
♂*	11	13.0-21.5	17.32±0.71

*Castrated.

It was noteworthy that although the net mean increase in the testes weight was only about 100%, the ovarian weight increment in all experiments was significant. Previous work[5] indicated that the bird ovary responded only to much greater dosages of gonadotropic hormone than the testes. Chick ovaries, therefore, are apparently much more responsive than those of other birds since these results with implants confirm similar findings which followed hormone injections.[2]

Preliminary histological study of the testes of birds that received male pituitaries showed stimulation of the seminiferous tubules and only slight increase in interstitial tissue. Testes of those chicks, however, which were given female pituitaries responded with only a slight increase in the tubules but with an hypertrophy

[5] Schockaert, J. A., cited by Engle, 1932, *Sex and Internal Secretions*, Baltimore, 778.

of the interstitial tissue. This hypertrophy was characterized by masses of compact, lightly staining cells which resembled, at least superficially, the "luteal cells" of the bird ovary. A more detailed cytological study is now in progress.

These experiments show that chick testes were only moderately stimulated by F.S.H. when such hormone was made available by implantation of pituitaries from castrate rats, and that female pituitaries elicited the greatest physiological response. Several factors may be suggested as influencing these results: (1) possible deleterious effect of the temperature which is about 5°F. higher in the bird; (2) greater weight of the female pituitary gland of the rat; and (3) failure to get an adequate "take" of the gland due either to place of implantation or to the fact that foreign protein was introduced. These variables are being investigated as far as possible.

8986 C

Tolerance of the Rhesus Monkey to Pituitrin Injections and Absence of Uterine Bleeding Following Injection.

CARL G. HARTMAN AND E. M. K. GEILING.

From Carnegie Laboratory of Embryology and Johns Hopkins School of Medicine and University of Chicago.

The following experiments were performed to test the possible rôle of pituitrin in the causation of menstrual bleeding. Incidentally the high tolerance of the Rhesus monkey to pituitrin was demonstrated.

By observing the vascular phenomena in intra-ocular transplants of the endometrium Markee[1] has shown that the most constant feature of menstruation is the constriction of the spiral arterioles of the functionalis layer. In view of the further finding of Hartman and Firor[2] that menstrual bleeding could be elicited in monkeys from which the anterior but not the posterior pituitary had been removed, it seemed reasonable to suppose that pituitrin might be responsible for the vasoconstriction that leads to necrosis and sloughing of the functionalis in menstruation.

Preliminarily, a number of female monkeys were injected with large doses of pituitrin to determine tolerance as well as the effect

[1] Markee, J. E., *Anat. Rec.*, 1936, **64**, 32.

[2] Hartman, C. G., and Firor, W. M., *Anat. Rec.*, 1935, **61**, 55.

of the drug on the sex color, which has also been shown to be a vascular phenomenon. Two experiments are cited.

Monkey No. 7, 15-year-old female having constantly red buttocks and nipples, shade No. 9 on a color scale of 10. Bleedings occurred on September 19, November 1, 1934, and January 31, 1935. On December 11, 1934, five units of pituitrin were injected intravenously. Clotting of blood was prevented by the use of a calculated amount of heparin. In 3 minutes the color of nipples and sex skin had dropped to 7; in 4 minutes to 6; in 7 minutes to 4. In 12 minutes it was back to 7.

Monkey No. 108, old female, buttocks constantly red—9 on a scale of 10. On December 11, 1934, day 7 of the cycle, 10 units of pituitrin were injected intravenously. The right and the left sides of the sex skin blanched at different rates, as follows: in 2 minutes, 7 and 6 respectively; in 3 minutes, 6 and 4; in 5 minutes, 5 and 3; in 6 minutes, 4 and 2. Although the injection was made into the right saphenus vein it was the left half of the sex skin that suffered the greater blanching.

No uterine bleeding followed such momentary action of the drug, nor was any expected, since Markee had determined that the period of continuous blanching lasts 6 to 20 hours before the menstrual bleeding begins. Accordingly 3 females were injected by a continuous "drop" method over a period of 5 hours in each case. A cannula was placed in the saphenus vein and connected with a burette by means of a thin-walled rubber tube. The pituitrin was dissolved in 100 cc. physiological saline and delivered to the blood stream at the rate of 3 cc. in one minute. The animals were anesthetized with Nembutal (Abbott), initial dose 0.4 grain per kilo body weight, supplemented when necessary with further injections of 0.25 grain by means of a hypodermic needle run through the rubber delivery tube. The animal was covered with toweling and the operating table was slightly warmed from below with an incandescent light bulb.

Non-ovulating females were selected in order to avoid the possible inhibiting action of progesterone on the reactivity of the arterioles.

I. *Rhesus Female No. 256;* weight 5200 gm.; injected on February 20, 1935, day 19 of the menstrual cycle, from 10:20 A. M. to 3:25 P. M. During this period the enormous amount of 464 units of pituitrin (232 mg.) was delivered. A total of 4.25 grains of nembutal was necessary to "hold" the animal, an amount considerably above the minimal lethal dose (about 0.6 grain per kilo)

if injected at one time. At 11:00 A. M. and at 2:14 P. M. the rectal temperature was 102.6°F., the pulse rate 170.

II. *Rhesus Female No. 217;* weight 4400 gm., very "regular" in menstruation though never ovulating. Bleedings January 16, February 11, March 7, April 3, 1935. Injected on March 5 (10:35 A. M. to 3:34 P. M.), with a total of only 4.4 units of pituitrin and 2.51 grains of nembutal. At 11:30 the rectal temperature was 100°F., the pulse rate 140.

III. *Bonnet Female (Macaca radiata) No. 147;* adult, castrated in February, 1932, body weight 3660 gm. She had received 100 R.U. estrin (Amniotin. Squibb) daily for more than 3 months until March 8, 1935. Injection of pituitrin was made on March 12 on the theory that 4 days after cessation of estrin injections uterine bleeding might be precipitated with the 15 units of pituitrin used. Bleeding did not occur until March 25.

In none of these injected animals was uterine bleeding hastened by the injections of 4.4, 15 or 454 units of pituitrin.

The usual anuria accompanied the injections.

8987 C

Virulence in Relation to Early Phases of the Culture Cycle.

FRIEDA OFFENBACH. (Introduced by C.-E. A. Winslow.)

From the Department of Public Health, Yale School of Medicine.

In view of the many physiological characteristics of bacteria which have been found to vary in different phases of the culture cycle, it was thought worth while to see if virulence for the animal host would also show correlation with "physiological youth."

A strain of *Salmonella enteritidis* (pathogenic for mice by mouth) was cultivated in a one per cent peptone medium continuously aerated with the apparatus devised in this laboratory by H. H. Walker. The lag-period of this organism in this medium was one hour and the period of logarithmic increase extended from the second to the sixth hour with decline setting in after the eighth hour.

Cultures in the first, fifth and sixth hours were used for the intra-abdominal injection of 95 mice, one cc. amounts containing either 10,000 or 100,000 organisms being employed. All mice died in periods varying from one to 15 days. The mean time of survival was as follows:

Organisms Injected	One-hour Cultures	5-hour Cultures	6-hour Cultures
	days	days	days
10,000	5.9	6.1	6.5
100,000	4.6	—	4.2

There seems no evidence of any appreciable difference in virulence in the early phases of the culture cycle.

8988 P

Endocrine Control of the Scrotum and a "Sexual Skin" in the Male Rat.

JAMES B. HAMILTON. (Introduced by H. E. Himwich.)

From the Departments of Anatomy and Physiology, Albany Medical School.

The "sexual skin" of the female is known to be under the control of estrogenic substances, particularly in primates. "Sexual skin" differentiation is much less clear cut in the male. The purpose of this preliminary article is to point out (1) that "sexual skin" exists in the male and is controlled by the male hormone, (2) that the development and maintenance of the scrotum is also dependent upon the male hormone.

In the adult male white rat the ventrocaudal portion of the scrotal sac is distinguished by its wrinkling and reddish-yellow color. This skin covers the region where a functional gubernacular ligament joins the caudal end of the epididymis to the cremasteric muscle. Proof of the endocrine control over this characteristically-pigmented and differentiated bit of skin and over the whole scrotal sac is adduced from the following:

(1) In 10 castrated and in 7 hypophysectomized adult male rats the "sexual skin" atrophied, the reddish-yellow hue faded, and in some cases a curled yellow layer sloughed off this region, exposing an underlying skin which was not grossly different from that of adjacent regions. The whole scrotum lost its pouch form and tended to resemble the condition in the female rat in that the skin of this region was bound rather closely around the pelvis and tail.

(2) Atrophy of specialized skin and atrophy of the scrotum as a whole are not a result of the removal of the mechanical weight and pressure of the testes. Replacement of the testes in 2 rats by paraffin pellets of weight, size and shape equivalent to the testes did not prevent post-castration atrophy. In one animal the scrotal skin became taut and cracked open over the "paraffin testis".

(3) Subcutaneous administration of testosterone acetate* in sesame oil prevented the post-castration and post-hypophysectomy degeneration of the sexual skin and of the scrotum in 14 rats.

(4) Twenty immature rats injected for 19 days, beginning at the age of 14 days, developed scrota 1½-3 times larger than those of uninjected litter-mates.

Investigations are now in progress testing (1) the amount of male hormone necessary for scrotal development and maintenance, (2) the comparison of cryptorchid and normal testes in producing scrotal growth, (3) the influence of the male hormone on the scrota of other animals besides the rat.

An endocrine control of the scrotum is of apparent significance in reproductive processes, in the treatment of sterility, cryptorchidism and other conditions. An undeveloped scrotum may prevent the proper descent of the testes. In the adult rat, hypophysectomy, with its resultant scrotal atrophy, causes return of the testes to an abdominal position.[1] Administration of the male hormone after hypophysectomy in the adult male rat results in at least temporary maintenance of the testes in the scrotum[1] and fecundity.[2, 1]

In view of these other[1] data it seems likely that the benefits obtained in cryptorchidism with luteinizing anterior pituitary-like substance[3] are due to the production of the male hormone. Several cases of cryptorchidism are now being treated with male hormone, but wide clinical use should be delayed until the effect of the potent testosterone acetate has been carefully evaluated, particularly with regard to a possible carcinogenic action upon the prostate. Such studies are now being made of the effect of testosterone acetate in rodents and in primates.

Summary. Development and maintenance of the scrotum and differentiation of a male "sexual skin" at the tip of the scrotum of rats is dependent upon the male hormone.

* Furnished under the trade-name Perandren by the Ciba Company.

1 Hamilton, J. B., and Leonard, S. L. To be published.

2 Nelson, W. O., *Science*, 1936, **84**, 230.

3 Werner, A., Kolling, D., Ellersieck, D., and Johns, G. A., *J. A. M. A.*, 1936, **106**, 1541.

8989 C

Some Unexpected Results in an Attempt to Produce Experimental Scurvy.

SIGMUND WEISS. (Introduced by Ivan E. Wallin.)

From the Department of Anatomy, University of Colorado School of Medicine, Denver.

Hay has been considered to contain little if any vitamin C (ascorbic acid) since Holst and Frölich[1] reported the production of experimental scurvy in guinea pigs using this substance as an aliment. Others, notably Hess and co-workers[2] have reported success in producing experimental scurvy in guinea pigs with a diet of hay, oats and water. Animals maintained on the latter diet, by the author, did not show any signs of scurvy, even after 3 months.

The major difference between the diet used by the author and that of the workers quoted above was in the variety of hay used. We fed the South Park variety of Rocky Mountain meadow hay, the main constituents of which are grass-like rushes and sedges. The predominant species are *Juncus columbianus, Carex arapahoensis* and *Carex siccata.* This type of hay has long been recognized by stock feeders as of superior nutritional quality because its protein and mineral ash content are higher than those of most other varieties of hay.

A series of experiments was designed to determine which constituent of the diet used contained the antiscorbutic agent. The first experiment was to substitute another grain for the oats. Rolled barley was chosen since it had been used in several scorbutic diets.[3,4] However, the guinea pigs maintained a steady growth on the diet of hay, rolled barley and water and showed no signs of scurvy. Since the change of grains (oats to barley) did not seem to make any appreciable difference, the hay was next studied. It was thought that heating the hay as much as 7 hours at 105°C. might oxidize any ascorbic acid that was present. The hay was placed in an asbestos oven, heated by an underlying gas burner. The oven has air holes at different levels, which allows a change of air during heating. Animals fed the *heated* hay, oats and water showed a rapid rise in weight throughout the experiment. They did not show any evidence of scurvy after one month of this regime. Evidently, even

[1] Holst, A., and Frölich, T., *J. Hyg.*, 1907, **7**, 634.

[2] Hess, A. F., *Scurvy Past and Present*, J. B. Lippincott Co., Phila., 1920.

[3] Harde, E., *C. R. Soc. de Biol.*, 1934, **116**, 153.

[4] Mouriquand, G., Weill, L., et Simon, F., *C. R. Soc. de Biol.*, 1934, **116**, 543.

with this amount of heating, the ascorbic acid was not destroyed completely. It was then thought, perhaps moisture was necessary to catalyze the oxidation of the vitamin C. To test this, hay was heated in the usual manner but a large pan of water was interposed between the hay and the source of heat. After 7 hours of heating at 110°C. all the water had not evaporated so that constant moisture had been present. On the diet of *moist heated* hay, oats and water, the animals did not gain in weight quite as rapidly as on the previous diet, but they showed no signs of scurvy after 7 weeks.

Further evidence that ascorbic acid is present in the hay used was determined in the following manner. Animals were placed on the basal ration suggested by Pelkan,[5] which has the advantage of being adequate in all nutritional factors with the exception of vitamin C. On this diet the guinea pigs showed signs of scurvy after the second week, and these signs were quite marked at the end of the third week. Another group of animals was put on the Pelkan diet plus *moist heated* hay, heated for 7 hours at 110°C. While both the last mentioned groups were started at the same time, the animals on the Pelkan diet plus moist heated hay continued to gain weight constantly and rapidly, showing no signs of scurvy; whereas those on the Pelkan diet alone lost weight rapidly after the onset of scorbutic symptoms.

Other suggestive evidence that ascorbic acid is present in the hay tested, is that a distilled water extract of the hay gives a strongly positive reaction with the reagent of Bezssonoff.[6] While it is true that the reagent of Bezssonoff is not specific for ascorbic acid, merely for di-enolic reducers, it is generally assumed that the only di-enolic reducer commonly found in plants and foodstuffs is ascorbic acid.

On the basis of the foregoing biological and chemical tests it seems probable that rather large amounts of ascorbic acid are present in the hay tested. The ascorbic acid present has not only withstood the oxidation inherent in the "curing" process of the hay, but also during the prolonged heating at 110°C. Yet this ascorbic acid may readily be extracted by distilled water.

These findings are diametrically opposed to those of Holst and Frölich, and Hess and co-workers. It appeared probable, therefore, that there might be a difference in the vitamin C content in different varieties of hay. To test this hypothesis 4 other varieties

[5] Pelkan, K. F., *Am. J. Dis. Child.*, 1925, **30**, 174.

[6] Bezssonoff, N., Delire, A., et Van Wien, H., *Bull. Soc. Chim. Biol.*, 1934, **16**, 1133.

of hay were tried. These were the Middle Park, North Park and Gunnison types of hay and alfalfa. It was possible to maintain guinea pigs in a good state of nutrition with any one of these varieties of hay without any signs or symptoms of scurvy.

It appears likely that some varieties of hay contain considerable amounts of vitamin C. Since some workers have used hay for roughage, with their basal diets, in the belief that hay contained little if any ascorbic acid, it was thought these results should be reported.

8990 C

Effect of Ascorbic Acid on Resistance of Suprarenalectomized Rats to Histamine.

David Perla.

From the Laboratory Division, Montefiore Hospital, New York City.

The removal of the suprarenal glands in rats is followed by a drop in resistance to certain poisons, to toxins and to bacterial and protozoan infections.* This drop is due essentially to the removal of the cortex, since the natural resistance may be raised almost to the normal by repeated injections of cortin.[2] With the discovery of the presence in large amounts of ascorbic acid in the cortex[3, 4] and its subsequent availability in synthetic crystalline form, an effort was made to determine the rôle of this factor in the resistance of suprarenalectomized animals. Szent-Györgyi had mentioned some years ago that it could not prolong the life of suprarenalectomized animals, nor did it influence the course of Addison's disease (except in modification of pigmentation).

Seventeen suprarenalectomized adult albino rats (3 months of age) were injected daily intraperitoneally with 10 mg. of ascorbic acid dissolved in physiological salt solution and brought to a pH of 7.5 immediately before injection. The injections were continued during a period of 8 days. On the 8th day, the 17 rats together with 14 untreated suprarenalectomized rats, all received

* For a survey of this subject, see the review by Perla and Marmorston.[1]

1 Perla, D., and Marmorston, J., *Arch. Path.*, 1933, **16**, 379.

2 Perla, D., and Marmorston-Gottesman, J., Proc. Soc. Exp. Biol. and Med., 1931, **28**, 650.

3 Szent-Györgyi, A., *Biochem. J.*, 1928, **22**, 1387.

4 Svirbely, J. L., and Szent-Györgyi, A., *Biochem. J.*, 1932, **26**, 865.

about one killing dose of histamine (ergamine acid phosphate) that is, 200 mg. per kilo of body weight. All the rats in both groups died within a few hours.

No protective effect against histamine poisoning was observed in suprarenalectomized animals repeatedly injected with an excess of ascorbic acid. It is probable that the altered natural resistance of suprarenalectomized rats is in no part due to a loss of ascorbic acid, nor can it be modified by an excess availability of the same.

Conclusion. It is suggested that the removal of the large store of ascorbic acid found in the cortex of the suprarenal gland is in no way responsible for the depression in resistance following suprarenalectomy.†

8991 P

Is Heparin an Antiprothrombin?

ARMAND J. QUICK.

From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee.

Heparin while not itself an antithrombin will react with an unknown substance in the plasma to form a strong antithrombin.[1] Obviously it is essential to eliminate or correct for this antithrombic reaction in any experiment designed to determine whether heparin can also act as an antiprothrombin. A simple means to accomplish this has been found. Eagle[2] has shown that by passing carbon dioxide through plasma diluted with distilled water, prothrombin with some fibrinogen and a small amount of other constituents are precipitated. This precipitate when dissolved and neutralized is readily converted to thrombin by the addition of calcium. If heparin is an antiprothrombin it should prevent this conversion. The details of the experiment and the results are as follows:

Ten cc. of oxalated human plasma were diluted with 100 cc. cold distilled water and carbon dioxide bubbled through for 10 minutes. The precipitate was removed by centrifugation, dissolved in 8 cc. of normal saline, neutralized to pH 7.0, diluted to 9 cc., and divided

† This is not, however, inconsistent with the evidence that vitamin C may play an important rôle in the natural resistance of certain animal species to infection. (See review on vitamin C and resistance by D. Perla and J. Marmorston, in press.)

1 Howell, W. H., and Holt, E., *Am. J. Physiol.*, 1918, **47**, 328.

2 Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 531.

into 2 equal portions. To the first were added 0.5 cc. heparin solution containing 5 mg. and 0.2 cc. of 0.1M calcium chloride, to the second, which served as control, 0.5 cc. saline and 0.2 cc. calcium chloride. A clot formed in both but appeared somewhat later in the solution containing heparin. After the removal of the fibrin, both were tested for their thrombic activity. 0.2 cc. of fibrinogen solution was mixed with 0.1 cc. of the thrombin solution and the clotting time determined. The reaction was carried out at a temperature of 39°-40°C. With this procedure, the coagulating potency of progressive dilutions of both thrombin preparations were determined. The results were:

	Clotting Time in Seconds				
Concentration of Thrombin	1	1/5	1/10	1/20	1/40
Thrombin Solution I (Heparin)	3	7.5	13	20	36
" " II (Control)	3	7.5	14	24	39

The fibrinogen was prepared from rabbit plasma. It was twice precipitated with half saturated sodium chloride and once with ammonium sulfate (quarter saturation). The heparin was prepared from beef lung by the method of Charles and Scott.[3] It had the same potency as the commercial product of Hynson, Westcott, and Dunning.

The results show that as much thrombin is formed in the presence of a large excess of heparin as in the control containing none. In this experiment heparin has no anticoagulating action, because neither the prothrombin preparation nor the fibrinogen contain the constituent with which heparin reacts to form an antithrombin. Since heparin does not appear to influence the conversion of prothrombin to thrombin, one must seriously question the commonly held view that it is an antiprothrombin. The author[4] has furthermore presented data to show that thromboplastin does not neutralize heparin. Experimental evidence suggests that heparin is neither an antithrombin nor antiprothrombin, but an antithrombogen, *i. e.*, an agent which reacts with a constituent in the plasma to form a true antithrombin.

[3] Charles, A. F., and Scott, D. A., *Trans. Roy. Soc. Canada*, 1934, **28**, Sec. V, 55.
[4] Quick, A. J., *Am. J. Physiol.*, 1936, **115**, 317.

8992 P

Retardation of the Gall Bladder in Pregnancy.

MAUDE M. GERDES AND EDWARD A. BOYDEN.

*From the Departments of Obstetrics and Anatomy, University of Minnesota.**

Following Mann and Higgins' pioneer study of the effect of pregnancy upon the emptying of the gall bladder in gophers,[1] several attempts have been made to appraise the efficiency of this organ in gravid women, but with contradictory results. The latter may be attributed to difficulties inherent in visualizing the gall bladder of pregnancy, to lack of quantitative methods of measuring the flow of bile and to absence of post-partum examination of individuals selected for study.

The present analysis is based upon intravenous cholecystography and upon computation of the changing volumes of the gall bladder after a standard meal.[2] During the last 2 years 21 gravid women have been subjected to this test. Curiously enough, in view of some of the reports in the literature, the gall bladder of only one of these failed to visualize. Three other series were discarded because of gall stones, vomiting or unsatisfactory shadows. Of the remaining 17 patients, 4 had been gravid 2 to 3 months, and thirteen 5 to 8 months. Five of the latter were also visualized post-partum.

Inspection of the mean curves of evacuation of the gall bladder in these groups shows that in the 13 women 5 to 8 months gravid (most of them primigravidae) only half the contents of the gall bladder had been discharged 40 minutes post-cibum, whereas in 12 nulligravidae of comparable age[3] nearly three-fourths of the contents had been emptied (Fig. 1). This retardation is even more striking when the gravid and post-partum curves of 5 of these individuals are compared. During pregnancy these 5 gall bladders discharged only 8% of their volume in the first 20 minutes post-cibum, whereas 6 to 9 weeks post-partum the same organs discharged 48%. It is not surprising, therefore, that Potter should have found that 75% of normal gall bladders during pregnancy are distended.[4]

The explanation for this partial stasis, with its accompanying increase in the concentration of bile constituents and its disturbance

* Aided by grants from the Medical Research Funds of the Graduate School.

1 Mann, Frank C., and Higgins, George M., *Arch. Surg.*, 1927, **15**, 552.

2 For methods, see Boyden, E. A., *Anat. Rec.*, 1928, **40**, 147.

3 Boyden, E. A., and Fuller, Alice H., *Am. J. Dis. Child.*, 1934, **48**, 565.

4 Potter, Milton G., *J. A. M. A.*, 1936, **106**, 1070.

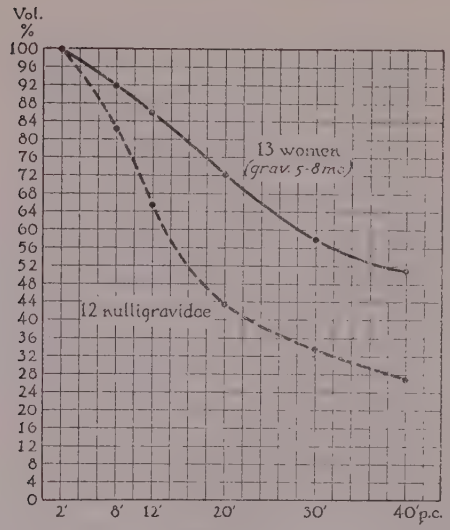


FIG. 1.

Mean curves of evacuation of gall bladder, after a standard meal of 4 egg yolks mixed in an equal volume of milk.

of bile salt-cholesterol ratios[4] cannot be adequately discussed within the limits of this preliminary article. Suffice it to say that Westphal's pilocarpine experiments on pregnant women,[5] pointing to a hypermotility of the Sphincter of Oddi during pregnancy, seems to afford the most promising line of investigation. Just when this delay in emptying becomes recognizable has not yet been determined. In the 4 individuals who were pregnant only 2 to 3 months the mean curve of evacuation of bile was only a little slower than normal—a difference that was not statistically significant.

8993 C

Kinetics of Muscle Atrophy in Different Species.

G. CLINTON KNOWLTON AND H. M. HINES.

From the Department of Physiology, State University of Iowa, Iowa City.

It has long been known that when skeletal muscle of homotherms is denervated, an atrophy ensues. The muscle undergoes a progressive loss of weight which is directly proportional to lapse of time after denervation. Quantitative data concerning the percent

[5] Westphal, Karl, *Z. f. Klin. Med.*, 1923, **96**, 95.

of weight lost by skeletal muscle at various periods of time after denervation have been reported by Langley and Kato,[1] Lipschütz and Audova,[2] and Chen, Meek, and Bradley.[3] The last authors noted that the curve relating weight loss to time was of a logarithmic type. No precise definition of the relationship between weight loss and time has, however, been given. It was suggested by Hines and Knowlton[4] that, in the rat, the loss of muscle weight in denervation atrophy follows very closely the curve of the equation for a reaction of the first order.

This idea has been tested on the results from experiments on rats, mice, guinea pigs, pigeons, and dogs. In each instance the gastrocnemius of one side was denervated and after designated periods of time the weights of the denervated and control muscles were determined. The weight loss of the denervated muscle was calculated on the basis of the assumption that the control muscle weight represented the initial weight of the denervated muscle.

It is immaterial whether the calculation is made on the basis of wet or dry weight since such slight alterations as occur in the water concentration of denervated muscle (Hines and Knowlton[4]) would not be of significance for these calculations.

The assumption of the identity in weight of 2 contralateral muscles from an animal is only true statistically. For any given animal it is not precisely true. Furthermore, this assumption is vitiated if the animal undergoes a significant change in body weight after denervation. If, however, the animals are fully grown and in a good nutritional state, the assumption is entirely justified.

With these facts in mind, the percent of weight lost during varying periods of atrophy was determined. The averages are shown in Table I. The test of fit of the individual weight loss data to the equation $k = 1/t \log_{10} A/(A-x)$ was made.

If the data fit the curve, "k" should be a constant characterizing the atrophy rate.

"t" is the atrophy time in days and is not equal to the time after denervation. Easily recognizable changes, such as fibrillary contractions, acetyl choline sensitivity, weight loss, and reduced glycogen concentration do not make their appearance until 48 to 72 hours after denervation. The peripheral stump of the cut nerve has lost its viability slightly earlier than this so that the atrophy time "t" is closely approximated by making it equal to the time, in days, after denervation minus 1½.

1 Langley, J. N., and Kato, T., *J. Physiol.*, 1914-15, **49**, 432.
2 Lipschütz, A., and Audova, A., *J. Physiol.*, 1921, **55**, 300.
3 Chen, K. K., Meek, W., and Bradley, H. C., *J. Biol. Chem.*, 1924, **61**, 807.
4 Hines, H. M., and Knowlton, G. C., *Am. J. Physiol.*, 1933, **104**, 379.

TABLE I.

Days after Denervation	No. of Animals	Weight Loss %		k*
		Rats.		
3	17	8.3		.030
5	10	16.4		.027
7	26	21.3		.023
14	22	48.3		.029
21	25	61.9		.030
28	23	72.3		.032
42	7	79.0		.029
Total	130		Mean k†	.029
Life Span[8] = 3.5 years.			P. E.	.0005
		Mice.		
3	19	6.3		.024
5	15	15.7		.025
7	19	22.5		.025
14	13	50.8		.032
21	12	68.4		.036
Total	78		Mean k†	.028
Life Span[9] = 2 years.			P. E.	.0009
		Guinea Pigs.		
7	6	18.4		.019
14	6	39.5		.022
21	4	54.3		.022
Total	16		Mean k†	.021
Life Span[8] = 6 years.			P. E.	.0009
		Dogs.		
28	4	29.8		.0071
42	5	17.3		.0025
118	5	40.0		.0024
132	7	54.8		.0036
Total	21		Mean k†	.0037
Life Span[8] = 15-20 years.			P. E.	.0003
		Pigeons.		
7	9	11.8		.012
14	9	28.8		.014
21	11	33.3		.011
28	4	37.0		.010
Total	33		Mean k†	.012
Life Span[8] = 12 years.			P. E.	.0006

*$k = \frac{1}{t - 1.5} \log_{10} \frac{85.5}{85.5 - x}$; t = time, in days, after denervation; x = % weight lost. The values of k are calculated from individual data.

†k calculated from the individual data.

[8] Abderhalden, *Handbuch der Biologischen Arbeitsmethoden*, Vol. V, Part 3 C.

[9] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1920, **42**, 71.

"A" is the percent of the original muscle weight which can be lost in atrophy and is taken as 85.5. There is no way to quantitatively determine "A", but all reports have indicated that the connective tissue, blood vessels, etc. (non-loseable portion) of muscle are between 10 and 20% of the total weight. The first estimate was taken as 85% for loseable weight and in testing the rat data to the equation, the fit was apparent. Applying the method of least squares,

it was found that 85.5% gave a better fit than 85%. This figure (85.5%) was then used throughout the calculations.

"x" represents the percent of original weight lost in time "t".

It should be noted that the values for "k" given in Table I are the means of the "k's" calculated from the individual weight loss data and are not calculated from the mean weight loss. Likewise, the mean "k" for a species is calculated from the array of individual "k's".

It is clear that the data, within a species, give a reasonably close fit to the curve suggested as evidenced by the constancy of "k" after different periods of atrophy (Table I). "k" seems to have a characteristic value for a given species. The only exception is the data on the dog.

Apparently a genetic factor is of importance in determining the atrophy rate. This probably accounts, in part, for the lack of uniformity in the results from dogs. The dogs used did not represent a genetically homogeneous sample. The other specie groups did. Another factor in the dog is the slower atrophy rate of the muscle, thus giving more opportunity for a regeneration of nerve so that continuity is often reëstablished, even after the removal of a fairly long nerve section, before marked atrophy occurs. All animals in which this regeneration had proceeded to the point of giving a functional nerve were of course discarded. However, the question arises as to the possible "trophic" effect of the embryonic type of tissue first produced in the regeneration of motor nerve.

With these mitigating factors present in the dog, it seems justified to disregard the data on this animal for the time being and conclude that the mean rate of denervation atrophy in a homogeneous population of homotherms is defined by the equation of the curve for a reaction of the first order.

It seemed of interest to attempt to relate the absolute index of atrophy rate, "k", to some other characteristics of an animal. Changes in metabolic rate due to different levels of thyroxinization have been shown to alter the rate of denervation atrophy weight loss in the rat (Hines and Knowlton[5]). Metabolic rate changes due to differences in environmental temperature did not alter the atrophy rate in the rat (Hines and Knowlton[6]). That differences in metabolic rate do not account for the different atrophy rates in different species is shown by a comparison of the "k" for mice and rats. The effective metabolic rate would, of course, be that per

[5] Hines, H. M., and Knowlton, G. C., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1029.

[6] Hines, H. M., and Knowlton, G. C., *Am. J. Physiol.*, 1934, **110**, 8.

gram of muscle. The mouse and rat have about the same metabolic rate per unit surface area, but the volume-to-area ratio is about 3 times as great in the rat as in the mouse. This means that the metabolic rate per gram of muscle must be about 3 times as great for the mouse as for the rat. In spite of this large difference, the atrophy rates are the same.

Logically, one might expect denervation atrophy rate to be related to growth rate. A comparison between rats and mice shows the same atrophy rate and approximately the same growth rate. Such a comparison between the other species of this series is not so simple because the physiological age at birth or hatching of rats, mice, guinea pigs, and pigeons varies considerably (Brody and Ragsdale[7]). Thus, their growth rates immediately after birth and at the times from birth to maturity vary to some extent merely on the basis of development at birth.

Another characteristic, related to growth rate and far enough removed from birth to make differences in development at birth of less importance, is the life span. On comparing the life span with the atrophy rate "k" (Table I), it will be seen that the longer life span is, in general, associated with a slower rate of denervation atrophy. It seems useless, at the present time, to attempt any more precise correlation between the life span and atrophy rate. Before that could be done, life tables for each of the species would have to be available. The populations of these experiments do not represent an average species group, since only adult individuals were used. Thus the life span should be greater for these animals than the average life span of the species.

It does seem, however, as if the specific denervation atrophy rate, "k" is an hereditary characteristic of a species, possibly related to the genetic factors associated with determinaters of growth rate.

Summary. The relationship of the rate of weight loss in denervation atrophy to time has been found to be defined by the equation

$$k = \frac{1}{t} \log_{10} \frac{A}{A - x}$$

where "k" is a constant characteristic of the species and probably related to growth rate, "t" is the time from loss of nerve viability, "A" is the percent of the original weight which is loseable in atrophy, and "x" is the percent of total weight lost at time "t".

[7] Brody, S., and Ragsdale, A. C., *J. Gen. Physiol.*, 1922, **5**, 205.

8994 P

Photodynamic Action of Methylene Blue on Pneumococcus.

T. T'UNG. (Introduced by S. H. Zia.)

From the Department of Bacteriology and Immunology, Peiping Union Medical College, Peiping, China.

In a previous study on the photodynamic action of methylene blue on bacteria[1] it was observed that such treatment exerted a lethal effect on pneumococci. Further work along this line revealed that, in addition to the lethal effect, this treatment has a preservative action on their morphology and staining property when they were kept at 37°C. Even after a period of 6 months, it was found that at the temperature mentioned, these organisms still retained their Gram positiveness comparable to fresh living cultures. On the other hand, the same organisms treated either with methylene blue alone or with formalin lost their Gram positiveness and showed signs of deterioration within a month. The procedure by which the above results were obtained is described below.

A virulent culture of pneumococcus type III grown in Avery's medium for 18 hours was mixed with a physiological saline solution saturated with methylene blue in a Petri dish in the ratio of 9 to 1. The total volume was 20 cc. The mixture was divided into 2 equal portions, one unexposed, and the other exposed to an electric light of 100 candle power at a distance of 10 cm. for a period of 45 minutes. The dish when exposed was put over a Frigidaire cooling machine which kept the temperature below 20°C. After exposure, the microorganisms were washed in physiological saline to remove the methylene blue. The samples were centrifuged and then resuspended in an equal volume of saline. They were incubated at 37°C. together with a control culture preserved with 0.4% formalin. Smears made from them were treated with alcohol for 2 hours prior to the application of Gram stain for examination giving the results stated above.

In view of the excellent preserving effect of the photodynamic action of methylene blue on pneumococcus, it was considered of interest to see whether or not pneumococci preserved by the photodynamic action of methylene blue would also show a higher antigenic power than those treated with formalin, the latter, according to Tao[2] preserved type I pneumococci better than heat and possessed a higher antigenic power.

---

1 T'ung, T., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 328.

2 Tao, S. M., *Chinese M. J.*, 1932, **46**, 12.

One series of 4 rabbits was, therefore, immunized with a pneumococcus type I vaccine subjected to the photodynamic action of methylene blue and another series with vaccine treated with 0.4% formalin. The method of preparation of these vaccines was the same as described above except a 10 times more concentrated culture was used. The vaccines were kept in the ice-chest and used soon after preparation. The rabbits were inoculated intravenously for 6 consecutive days, followed by a week without inoculation. The same course of treatment was repeated twice, making a total of 18 injections representing 9 cc. per animal. Ten days after the last inoculation, the animals were bled and agglutination tests carried out. The 4 rabbits inoculated with pneumococcus vaccine subjected to the photodynamic action of methylene blue yielded sera which gave complete agglutination for 2, to a titer of 1:128, and one each to 1:32 and 1:16, whereas, the other 4 rabbits immunized with formalinized pneumococcus vaccine gave sera that resulted only in partial agglutination at titres not higher than 1:16.

In addition, protection test on white mice was also carried out, using the same methylene blue and formalin treated vaccines of pneumococcus type I. Two series of 30 white mice each were inoculated intraperitoneally with 0.5 cc. each of these 2 vaccines respectively every other day for 4 times. Eighteen days after the last inoculation, they were injected with 0.5 cc. of different dilutions of an 18-hour culture of the same strain of pneumococcus. The animals were kept under observation for 7 days and cultures were made from the heart blood of all those that died. It was found that the control animals inoculated with the test cultures were killed up to a dilution of 1-1,000,000, while the vaccine prepared by the photodynamic action of methylene blue protected a majority of the mice up to a dilution of 1-10 of the virulent culture. On the other hand, the formalinized vaccine protected the mice to a dilution of 1-1,000. This observation shows that type I pneumococci subjected to the photodynamic action of methylene blue are distinctly more antigenic than those treated with formalin. Further work on the protective and antigenic action of this particular vaccine to different types of pneumococcus is under investigation.

8995 C

Effect of Cocaine upon Protein Content of Regenerated Aqueous Humor.

PETER C. KRONFELD AND C. K. LIN.

From the Department of Ophthalmology, Peiping Union Medical College, Peiping, China.

The normal aqueous humor resembles in its composition a dialysate of the blood plasma with the normal capillary wall acting as dialyzer. When this aqueous is removed a "second" aqueous is formed which in animals contains considerably more protein than the original fluid. This second or regenerated aqueous is generally thought to be formed by filtration of plasma through abnormally permeable capillary walls. If the paracentesis is done after retrobulbar injection of adrenaline or during stimulation of the sympathetic, the new-formed aqueous contains only slightly more protein than the normal one. In man the difference between the intraocular fluid of new formation and the normal fluid is less marked. Some writers have suggested that there is no increase in the protein content of the human aqueous after paracentesis. More accurate techniques have demonstrated that an increase of protein, although small, does occur (Table I). Two factors have been suggested as possibly having some influence upon the protein content of the second aqueous, *viz.*, the time interval between the withdrawal of the first and the second aqueous and the anesthetic used. Mestrezat and Magitot[1] conclude from their small series of cases that the protein content of the regenerated aqueous in man reaches its maximum about 45 minutes after the first paracentesis. Wessely[2] has pointed out that anesthetics with pronounced vasoconstrictor action like cocaine are likely to lower the protein content of the second aqueous. He has, however presented no proof for this view.

We have had the opportunity to determine the protein content of the first and second aqueous on a small number of practically normal human eyes. Under local anesthesia the aqueous was aspirated by needle puncture and its protein content determined by nephelometry of the opacity produced by precipitation of the protein with sulphosalicylic acid.[3] The second paracentesis was always done from 45 to 65 minutes after the first one, so as to strike the alleged maximum of the protein content.

---

1 Mestrezat, W., and Magitot, A., *Annales d'oculistique*, 1922, **159**, 401.

2 Wessely, K., *Arch. f. Augenheilkunde*, 1923, **93**, 194.

3 Franuschetti, A., and Wieland, H., *Arch. f. Augenheilkunde*, 1928, **99**, 1.

TABLE I.

Author	Clinical Diagnosis		Protein Content of the First (I) Aqueous	Protein Content of the Second (II) Aqueous	Increase in Protein Content II/I	Interval	Anesthetic
Franuschetti and Wieland	Senile cataract		.021	.097	4.6	23 min.	Cocaine, 2%
	,, ,,		.034	.080	2.35	30 ,,	,,
	,, ,,		.027	.070	2.6	15 ,,	,,
	,, ,,		.030	.140	4.7	15 ,,	,,
Wessely	Keratectasia		.010	.080	8		Holocaine
	Optic atrophy		<.01	.02	at least 2	1½ hrs.	,,
	,, ,,		.01	.06	6		,,
	,, ,,		.01	.03	3	30 min.	Cocaine
Gilbert	Strabismus, Amblyopia		.015	.175	12	1 hr.	Apocaine
	Optic atrophy		.017	.119	7		?
	Active choroiditis				6-8		?
Dieter	Aphakia		.010	.062	6	73 min.	?
	Cataract		.014	.08	6	1 hr.	?
	,,		.024	.07	2.5	1 ,,	?
	,, , Diabetes		.018	.083	4	48 min.	?
Mestrezat and Magitot	Neuritic optic atrophy	R.E.	.01	.025	2.5	3 hr.	Novocaine
		L.E.	.01	.150	15	1 ,,	,,
		R.E.	.01	.085	8.5	1 ,,	,,
	Toxic ,, ,,	R.E.	.01	.075	7.5	1 ,,	,,
		L.E.	.01	.200	20	40 min.	,,
	Tabetic ,, ,,	R.E.	.012	.050	4	25 ,,	,,
		L.E.	.015	.120	8	30 ,,	,,
	,, ,, ,,		.023	.070	3	30 ,,	,,
	,, ,, ,,		.030	.110	4	45 ,,	,,
	,, ,, ,,		.023	.240	10	45 ,,	,,

Our results for the protein content of the normal and the regenerated aqueous were not essentially different from those reported by other authors. The difference in protein content between the second and first aqueous was, however, as a rule more pronounced, which may be a characteristic of the eye of the Chinese. We were chiefly interested in proving or disproving Wessely's view of the rôle played by the local anesthetic. We therefore determined the protein content of the first and second aqueous on 4 eyes of 3 patients twice, the first time using an anesthetic without vasoconstrictor action (pantocaine 1%, butyn 2%) and the second time using cocaine (10%). From 8 to 10 drops of the anesthetic were instilled before each puncture.

TABLE II.

Age	Clinical Diagnosis		Protein Content of the First Aqueous (I)	Protein Content of the Second Aqueous (II)	Increase in Protein Content II/I (approximately)	Anesthetic
20	Bilateral retrobulbar	L.E.	.0070	.270	40	Butyn
20	neuritis	R.E.	.0077	.149	18	"
16	Nutritional edema,	R.E.	.0057	.181	32	Pantocaine
16	normal eyes	L.E.	.0093	.174	19	"
22	Retrobulbar neuritis		.011	.122	11	"
38	Tetanic cataract		.0071	.479	68	"
11	Tumor of the maxilla, normal eyeball		.0080	.533	66	Butyn

TABLE III.

Protein Content of the First Aqueous	Protein Content of the Second Aqueous	Increase in Protein Content II/I (approximately)	Anesthetic
.0120	.0535	4	Cocaine
.0081	.0368	4	"
.0056	.022	4	"
.0112	.0686	6	"

The results given in Table III indicate that cocaine had a definite effect upon the protein content of the regenerated aqueous. The amount of fluid present in the anterior chamber at the time of the second puncture and consequently the rate of fluid production seemed to be independent of the type of anesthetic used. The effect of cocaine upon the protein content of the regenerated aqueous may be due to its vasoconstrictor action and/or—because of its greater and deeper reaching anesthetizing power than that of any of the other anesthetics used—due to the inhibition of axon-reflexes. The effect of other drugs upon the protein content of the regenerated aqueous in man is being studied.

8996 P

Decurarizing Effect of Repetitive Stimulation of a Motor Nerve.

T. E. Boyd and John J. Brosnan.

From the Department of Physiology and Pharmacology, Loyola University School of Medicine.

One of us[1] described a temporary return of indirect excitability in the tongue of the curarized cat, following tetanization of the hypoglossal nerve. We have made some additional observations on the phenomenon, using the cat's peroneal nerve and tibialis anticus muscle. The experimental conditions were as described in our accompanying paper,[2] except that crude curare (1% solution) was administered instead of $MgSO_4$.

Curarization was carried to the stage at which single stimuli, applied to the otherwise resting nerve, failed to elicit a muscular twitch. The nerve was then tetanized, for periods varying from 1 second to one minute, the frequency also being varied from 14 to 120 per second.

Comparing the results with those obtained on animals "curarized" to the same stage with Mg^2, certain similarities appear. In both conditions, (a) tetanization of the nerve is followed by a return of the response to single stimuli; (b) the degree of this recovery varies with the frequency of tetanic stimuli employed; (c) the duration of the recovery is about the same, following a given period of stimulation.

Under curare, however, the first twitches obtained during the recovery are relatively weak. The curve described by the successive twitches shows first a rising and then a longer falling phase. Following 5 seconds of stimulation of the nerve, at 120 per second, the maximum recovery is seen in 6 to 10 seconds. If the period of tetanization is lengthened to 20 seconds the entire recovery curve is stretched out, so that the rising phase lasts for half a minute or more.

It seems to have been proven earlier[1] that this recovery is produced through the motor innervation of the muscle, and not through other fibers in the mixed nerve. If we attribute it to a chemical decurarizing agent, released at the nerve endings, the curve described seems to indicate that the concentration of the agent increases for a time after activity of the nerve has ceased. Acetyl

[1] Boyd, T. E., *Am. J. Physiol.*, 1932, **100**, 569.

[2] Brosnan, J. J., and Boyd, T. E., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 405.

choline, accumulating in excess, might have a depressing effect, although this action of acetyl choline is said to be reversed under curare.[3, 4] The delayed recovery here is evidently not so caused, for acetyl choline (1 mg. in 1 cc.) injected intra-arterially during the rising phase of the recovery curve, has an immediate potentiating, not a depressing, effect.

Nevertheless repetitive stimulation of the nerve, under curare, does appear to leave some kind of after-depression which is not found with Mg. No muscular response was ever obtained *during* tetanization of the nerve, even with frequencies as low as 14 per second (*Cf.* Bremer and Titeca[5]). The recovery curve may be determined by 2 factors, the changing concentration of a decurarizing chemical and gradual recovery from a depression which varies in depth and duration according to the previous activity of the nerve. Unless the latter factor is assumed to exist, the decurarizing agent appears to be too slowly mobilized to be concerned in the normal excitation of muscle.

8997 P

Chemical Transmission from Nerve to Muscle, in Animals "Curarized" with Magnesium Sulphate.

JOHN J. BROSNAN AND T. E. BOYD.

From the Department of Physiology and Pharmacology, Loyola University School of Medicine.

Lubinska[1] has described a type of peripheral summation or facilitation in cats. Following the administration of sufficient $MgSO_4$ intraperitoneally, the muscular response to single stimuli applied to a motor nerve is lost. Repetitive stimuli, however, evoke a tetanus, and temporarily thereafter single stimuli are again effective.

It seems to us that such effects might be due to local accumulation of some decurarizing chemical agent released at the nerve endings. Acetyl choline[2] and potassium[3] are temporarily effective antagonists

3 Rosenblueth, A., Lindsley, D. B., and Morison, R. S., *Am. J. Physiol.*, 1936, **115**, 53.

4 Briscoe, Grace, *J. Physiol.*, 1936, **87**, 425.

5 Bremer, F., and Titeca, J., *Arch. Int. Physiol.*, 1935, **42**, 223.

1 Lubinska, L., *Arch. Int. de Physiol.*, 1935, **41**, 456.

2 Rosenblueth, A., Lindsley, D. B., and Morison, R. S., *Am. J. Physiol.*, 1936, **115**, 53.

3 Wilson, A. T., and Wright, S., *Quart. J. Exp. Physiol.*, 1936, **26**, 127.

to curare, and it has been inferred that both substances are released at the endings of cholinergic nerves.[4, 5]

We have used cats and dogs, anesthetized with nembutal. Contractions were recorded from the tibialis anticus muscle, the lever allowing 1.1 mm. of shortening per kilogram of tension. The sciatic trunk was sectioned and shielded electrodes placed on the peroneal branch. Single test stimuli, from a neon-tube circuit, were applied at intervals of 4 to 6 seconds. For tetanizing we used a photocell stimulator with a rotating disc interrupter. Both circuits were adjusted so that the shocks were barely strong enough to give a maximum normal twitch. $MgSO_4$ (7.7% solution of the heptahydrated salt) was administered intravenously in small repeated doses.

When response to single shocks had failed, it was always restored following a period of tetanic stimulation of the nerve. The higher the frequency of tetanic stimuli (up to 120 per second) the greater the degree of recovery after a short tetanus. The "physiological" frequency of 27 per second, used by Lubinska, is therefore not an optimum for this effect. The duration of the recovery varies both with the frequency and with the duration of the preceding tetanus. Following a tetanus of 30 seconds at 120 per second, the restored twitches may take 8 minutes or more in sinking to zero or to a new base level. If this recovery is due to accumulated acetyl choline, the local mechanism for its removal is obviously slow in action.

Recovery of the response to single shocks is at a maximum immediately after cessation of the tetanus. The twitches fall away rapidly at first and then more slowly, describing a smooth curve. Such a curve would be expected if the diminishing concentration of a chemical activator were plotted against time, but it is not apparent why the twitch-tension should follow the same type of curve. An entirely different form of recovery curve is obtained in animals under curare.[6]

$CaCl_2$ (2 cc. of isotonic solution) or physostigmin (0.5 to 1 mg. per kilo) injected into the iliac artery, induce a gradual but sustained recovery. Acetyl choline (0.2 to 2 mg. in 2 cc.) and KCl (1 to 3 cc. of 2 to 4% solution) give recovery curves which more nearly resemble those following brief tetani.

---

4 Dale, H. H., Feldberg, W., and Vogt, M., *J. Physiol.*, 1936, **86**, 353.

5 Feldberg, W., and Guimarais, J. A., *J. Physiol.*, 1936, **86**, 306.

6 Boyd, T. E., and Brosnan, J. J., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 404.

8998 P

Mechanism of Cobalt Polycythemia. Effect of Ascorbic Acid.

ALBERTO GUZMAN BARRON AND E. S. GUZMAN BARRON.

From the Lasker Foundation for Medical Research and the Department of Medicine, University of Chicago.

Waltner and Waltner[1] discovered that cobalt when given to animals produced a polycythemia as shown by the increase in the number of red cells and in hemoglobin. Mascherpa[2] from studies of cobalt polycythemia on dogs reported an increased activity of the bone marrow. For experiments on the mechanism of this polycythemia rabbits were used so that blood could be withdrawn in sufficient quantities to measure the hemoglobin concentration and count the red cells simultaneously. By daily subcutaneous injection of 0.01 gm. $CoSO_4$ a definite polycythemia was produced within 6 or 7 days, accompanied by the appearance in the circulating blood of reticulocytes and erythroblasts. The presence of these young cells was strikingly manifested by the increased respiration of red cells from animals with cobalt polycythemia, an increase which fails to appear in human polycythemia vera (Table I). The increase in respiration was about 10 times as great when erythroblasts were present.

TABLE I.

O_2 Consumption of Red Cells from Polycythemia.

pH, 7.38; T., 37° C. Figures were calculated for blood containing 5×10^6 red cells per cmm. 2 cc. per vessel.

Kind of Blood	O_2 Consumption cmm. per hour	Reticulocytes %	Erythroblasts %
Rabbit			
Control	4.3	2.06	—
Co polycythemia	11.9	9.27	—
" "	45.0	6.66	4
Man			
Polycythemia vera	5.3	0.2	—

The addition of $CoSO_4$ (0.01 mg.) *in vitro* to the red cell suspensions from cobalt polycythemia was followed by a marked inhibition in respiration as contrasted with the lack of such effect in the red cells of normal rabbits. This inhibition was practically confined to the respiration of immature red cells, being greater in the bone marrow than in the spleen and kidney (Table II).

[1] Waltner, K., and Waltner, K., *Klin. Woch.*, 1929, **8**, 313.

[2] Mascherpa, P., *Arch. ital. biol.*, 1930, **82**, 112.

TABLE II.

Kind of Tissue (Rabbit)	O_2 Consumption—cmm. per hour		
	Before	After Addition of $CoSO_4$	Inhibition %
Red cells (Co polycythemia)	10.7	3.9	63.6
" " " "	14.4	1.1	92.0
" " " "	50.2	5.1	89.8
" " normal	4.4	4.8	none
Bone marrow	20.8	12.9	38
Spleen	27.5	25.0	9.1
Kidney	41.5	36.7	8.9

The findings of Berwald, Arsenau, and Dooley[3] that cobalt fails to produce polycythemia in splenectomized rats did not hold in the case of rabbits, a discrepancy which may be explained by the fact that splenectomized rats develop an anemia by *B. muris*.

Ascorbic acid seems to assist in the maintenance of a determined level of red cells in the circulating blood, this effect in certain cases of anemia having been reported many times. When ascorbic acid was injected intravenously into the rabbits (60 mg. daily) simultaneously with $CoSO_4$, polycythemia failed to appear; when the ascorbic acid was withdrawn it appeared at the end of 6 or 7 days. When ascorbic acid was injected after the production of polycythemia a decrease in the hemoglobin concentration and red cell count resulted,

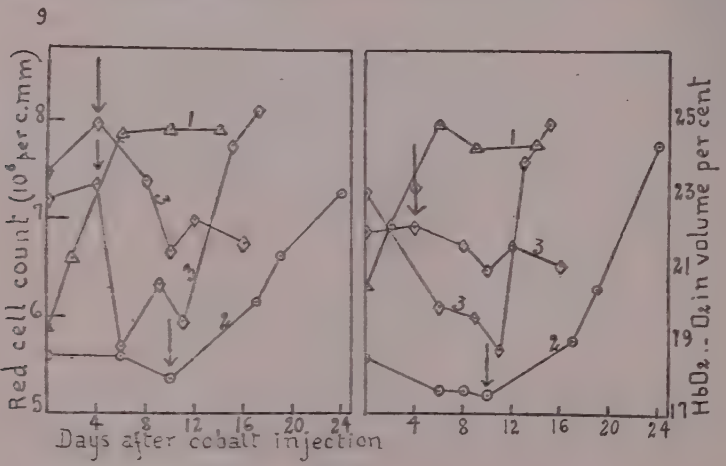


FIG. 1.

1. Control. Injection of $CoSO_4$ alone.
2. $CoSO_4$ and ascorbic acid injected simultaneously. 10 days after (marked by arrow) the ascorbic acid was withdrawn.
3. Cobalt polycythemia treated with daily injections of ascorbic acid.

[3] Berwald, W. P. E., Arsenau, J. H., and Dooley, M. S., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 430.

although the effect was temporary (Fig. 1). The concentration of hemoglobin ran in all experiments parallel to the red cell count.

According to these experiments cobalt polycythemia seems to be due to the inhibition by cobalt of the respiratory function of immature red cells. Once these cells have lost their ability to respire they are thrown into the general circulation as mature non-respiring cells, being replaced in the bone marrow by new cells. The function of ascorbic acid as one of the regulators of the level of red cells in the circulating blood seems probable in the light of these experiments.

8999 C

Participation of Ovarian Factors Other than "Estrin" in the Estrus Phenomenon.

S. C. FREED, T. GARVIN AND SAMUEL SOSKIN.

From the Department of Metabolism and Endocrinology, Michael Reese Hospital, Chicago, Illinois.*

We have recently shown that the evaluation of the potency of estrogenic substances cannot be based solely on the ability of these substances to cause vaginal cornification in test animals. Other physiologic effects, perhaps more important from a therapeutic standpoint, must also be considered.[1] However, even if the various activities of the known estrogenic substances were available to a high degree in a single "estrin", it would still be doubtful whether this estrin could serve therapeutically as a perfect substitute for the normal ovary, in bringing about the estrus phenomenon.

Aside from teleological reasoning based on its site of origin, the estrogenic substance elaborated by the ovarian follicle† merits consideration as being the true estrus hormone chiefly because its administration to castrate animals results in a state which resembles spontaneous estrus in the intact animal. With neither this nor any other estrogenic substance, however, has it been possible to reproduce in castrate animals a certain phase of estrus which has been

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

1 Freed, S. C., and Soskin, Samuel, *Endocrinology*, 1936, **20**, 863.

† Recently identified as dihydroxyestrin.[3]

3 MacCorquodale, D. W., Thayer, S., and Doisy, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1182.

observed in intact animals. We refer to the tremendously enlarged uteri in parabiotic rats, where one partner has been castrated.[2] In such an experiment the constant elaboration of follicle stimulating hormone by the pituitary of the castrate animal influences the ovaries of its partner to bring about a continuous estrus. Since there is no evidence that any pituitary substance can produce the above uterine response without the intermediation of the ovary, and since it has been impossible to evoke this response with the known estrogenic substances in the absence of the ovary, it seems necessary to postulate an ovarian factor other than or complementary to estrin, as being the responsible agent.

In view of the above, we were interested in comparing the results of prolonged estrin administration in normal and in castrated adult female animals. Our results indicate the existence of ovarian factors other than estrin, which affect the uterus in both an augmentative and inhibitory manner.

Methods and results. 25 International Units of dihydroxyestrin benzoate‡ were administered daily to a number of normal and castrated adult female rats. After periods of 4, 20, 30 and 60 days respectively of this daily treatment, about 10 of the normal and a similar number of the castrate animals were sacrificed for examination. The results are summarized in Table I.

Although vaginal cornification was present throughout the course of injections in both normal and castrate animals, examination of the uteri showed marked differences between the 2 types of animal, and in each type of animal at different times. Thus the uterus in the castrate animals reached its maximum weight after about 20 days of estrin treatment. Continued injections gave no further response, but rather some retrogression. The normal uterus, on the other hand, was not affected by the estrin treatment up to the 20th day. It then rapidly increased in weight and continued to do so throughout the time that the injections were maintained.

On closer examination the uteri showed even more marked differences. After 4 days of estrin administration, the uteri in the castrate animals were hyperemic and distended with fluid; the uterine epithelium was very high columnar, of the secretory type—the typical estrus reaction. After the same period of estrin treatment, the endometrium in the normal rats varied from the low cuboidal to high columnar. Only 2 out of the 10 normal animals presented

2 Martins, T., and Rocha, A., *Endocrinology*, 1931, **15**, 421.

‡ We are indebted to Dr. Gregory Stragnell of the Schering Corporation for a supply of this material.

TABLE I.

Effect of Daily Estrin Administration on the Uterus of Normal and of Castrated Adult Female Rats.

Days of Estrin Administration	Normals			Castrate		
	No. of Animals	Aver. Wt. of Uterus,* mg.	Description	No. of Animals	Aver. Wt. of Uterus,* mg.	Description
0	12	382	Different stages of estrous cycle	9	122	Atrophic
4	10	386	As above	14	316	Thin-walled; distended with fluid; advanced endometrial proliferation
20	8	390	,, ,,	8	420	Thick-walled; moderate endometrial proliferation
30	8	496	(a) 6 animals—thick-walled, moderate and advanced endometrial proliferation. (b) 2 animals—hypertrophied wall; distended with fluid; advanced endometrial proliferation.	8	384	As above
60	9	570	(a) 6 animals—as in (a) above (b) 3 animals as in (b) above	6	360	,, ,,

*Where distension is noted, uteri were weighed after removal of fluid.

uteri typical of estrus, others being in various stages of diestrus. The distribution of results in these normal animals differed in no way from that found in examining a similar number of untreated control rats.

After 20 days of estrin administration, the uterus in the castrate animals was pale and thick-walled. The uterine epithelium was not so tall as in the preceding stage. At this time the uterus in the normal animals appeared to be still unaffected by the estrin injections, various stages of endometrial development being found, as before. The ovaries, however, had undergone considerable atrophy.

At the end of 30 and 60 days of treatment, the uterus in the castrate animals was similar to that found at the 20-day interval. In the normal animals, however, the uterus had undergone a significant hypertrophy. Its diameter averaged twice that of the treated castrate uterus at the corresponding time. Five out of the 17 normal animals which comprised the 30- and 60-day groups had greatly enlarged uteri due to distension with fluid as well as thickening of the wall. The uterine endometrium in all the normal animals was now definitely proliferative, to a degree equal or surpassing that of the castrate uterus at this stage. By the 30th day, the ovaries had become extremely atrophic.

Our results indicate that the animals retaining their ovaries differ from castrate animals, in their uterine response to prolonged estrin administration, in at least 3 respects. The ovaries, therefore, presumably elaborate a factor or factors concerned with the following reactions (in order of their appearance in our results):

1. Preliminary inhibition of the uterine response to estrin.
2. Rhythmic activity of the endometrium.
3. Augmentation of the uterine response to estrin.

On first thought the remarkable resistance of the normal uterus for the first 20 days of estrin administration does not seem difficult of explanation. Since infantile animals resemble the castrate adults in their response to estrin, it is apparent that the refractoriness of the uterus in the normal adults must be due to some element in the mature ovary. It might seem reasonable to assume that the administered estrin stimulates the hypophysis to liberate its luteinizing hormone[4]; the corpora lutea are activated, giving rise to progestin, which antagonizes the action of the estrin. On closer examination, however, this explanation is not satisfactory. The simultaneous administration of progestin with estrin has been shown to prevent cornification of the vagina and to inhibit estrus in the

[4] Fevold, H. L., Hisaw, F. L., and Greep, J. R., *Am. J. Physiol.*, 1936, **114**, 508.

uterus, although it does not prevent the enlargement and maturation of the uterus.[5] In our work, on the contrary, the vaginal cornification was not inhibited and the uterus failed to develop.

The cyclic activity of the uterine endometrium in the normal rats throughout the 60 days of estrin administration, stands in sharp contrast to the relatively stable condition of the endometrium in the castrate animals under the same conditions. Since both types of animals were receiving the same ample supplies of estrin, it seems evident that the ovary must supply the factor responsible for the endometrial rhythm. And since, after the 20th day at least, the follicular apparatus of the ovary is extensively damaged, it seems likely on anatomical grounds that the rhythm factor originates in the interstitial tissue. In this connection, it is of interest that an extract of interstitial tissue recently has been shown to possess peculiar estrogenic properties[6] which are, as yet, difficult to correlate with our observations. But whether the rhythm factor is an estrogenic substance or a supplementary material having no estrogenic properties by itself, it is apparent that it is not included in the estrin of follicular origin.

The greater hypertrophy of the uterus in the normal animals during the later stages of our experiments, like the endometrial rhythm, can hardly be ascribed to the atrophic follicular apparatus. It is, of course, impossible to judge at present as to whether this augmenting factor is identical with the rhythm factor, whether it is an estrogenic substance more potent than those hitherto observed, or whether it is a substance which supplements the activity of the follicular estrin. In this regard, it may be noted that a very potent new, crystalline estrogenic substance which differs chemically from the known estrins, has recently been isolated from the hog's ovaries.[7]

Our results offer ample reasons to explain the failure of the castrate animal which is receiving estrin to reproduce the gigantic uterus of the parabiotic animal in continuous estrus. We have ascribed this failure to the lack in the castrate of ovarian factors which are available to the parabiotic animal. It may be that the lack of these same factors is responsible for the poor therapeutic results in certain cases, when estrin is administered as substitutional therapy to the human female. We are well aware of the possibility that the removal of the ovaries may result in a secondary disturbance in function of other organs associated with sex activity, and

[5] Selye, H., Browne, J. S. L., and Collip, J. B., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 198.

[6] Marlow, H. W., *Endocrinology*, 1936, **20**, 339.

[7] Andrew, R. H., and Fenger, F., *Endocrinology*, 1936, **20**, 563.

that our results may be due to these secondary disturbances rather than the lack of our hypothetical factors. Nevertheless the evidence for the latter seems to us to be sufficiently strong to warrant a careful search, in the hope of adding some useful agents to our endocrine armamentarium.

Conclusion. The effects of prolonged daily administration of estrogenic substance to normal and to castrated adult female rats are compared. It is concluded that, in the rat, "estrin" alone cannot completely replace the ovary in the induction of the uterine changes of the estrus phenomenon. Our results indicate the existence of ovarian factors other than estrin (or progestin) which influence the estrus phenomenon in at least 3 respects: 1. Preliminary inhibition of the uterine response to estrin. 2. Rhythmic activity of the endometrium. 3. Augmentation of the uterine response to estrin.

9000 C

Effect of Administration of Parathyroid Extract on Serum Calcium Level in the Nephrectomized Rat.*

WILBUR R. TWEEDY AND EDWARD W. MCNAMARA.

From the Department of Physiological Chemistry, Loyola University School of Medicine, Chicago.

Collip, Pugsley, Selye and Thomson[1] have observed resorption of bone in bilaterally nephrectomized rats which had been injected with massive doses of parathyroid extract. They are of the opinion that the primary action of parathyroid hormone is to cause an increased proliferation of osteoclasts, which actively function to release calcium from the bones to the blood. Since the observations of these workers were not supplemented by serum calcium determinations, which should yield evidence of the passage of calcium into the blood, we have carried out similar experiments† in which calcium analyses have been made.

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

1 Collip, J. B., Pugsley, L. I., Selye, H., and Thomson, D. L., *Brit. J. Exp. Path.*, 1934, **15**, 335.

† The incisors of the animals will be examined by Dr. Isaac Schour, and the femurs will be studied by Dr. F. A. McJunkin. Their findings will be reported in later communications.

Immature and mature rats of both sexes have been used. The animals (Table I) were not given food, but were supplied with water *ad libitum* during the 48-hour experimental period.‡ Parathyroid extract (Lilly)§ was injected immediately after nephrectomy, and successive doses were administered after 6, 12, and in some instances, 18 hours. Blood was obtained by cardiac puncture, without using an anesthetic, just before the animals were killed. Calcium analyses[2] were made by one of us (W.R.T.) on individual samples, which in most instances consisted of 2 cc. amounts of serum, and in no case less than 1 cc.

TABLE I.

Effect of Parathyroid Extract on the Serum Calcium Level in Normal and in Bilaterally Nephrectomized Rats.

Group	No. of Rats	Weight, gm.	Parathyroid Extract (Hanson)* units	Sacrificed after hrs.	Serum Calcium mg. per 100 cc.		
					Lowest	Highest	Average
			Normal Animals.				
A	12	86-141		48	9.36	12.52	11.14
B	5	94-122	250x2	48	13.92	16.68	15.05
	5	58-63	250x2	24	14.70	15.48	15.02
	2	227-245	250x3	48	12.87	16.68	14.78
			Nephrectomized Animals.				
C	14	62-287		48	8.72	11.93	10.53
D	6	98-127	250x4	48	10.48	13.64	12.09
	2	135-152	500x3	48	11.75	12.64	12.19
	3	152-269	250x4	48	8.95	11.32	10.18

*One "Hanson unit" is equivalent to one-fifth of a Parathormone (Collip) unit.

The data in Table I show that the serum calcium level in the normal rat (Group A) is well maintained during the first 2 days of starvation. After nephrectomy (Group C) the serum calcium level has a tendency to drop, and during the same period we have found in other work[3] that the serum inorganic phosphate increased rapidly. When the calcium values obtained in Groups B, C, and D are compared it is observed that in Group D there is only slight indication of mobilization of calcium as a result of parathyroid hormone administration. In no instance was definite hypercalcemia (values above

‡ The diet preceding the starvation period consisted of fox chow (commercial preparation) supplemented by cabbage, cheese, and lean meat once a week.

§ The authors are indebted to Eli Lilly and Company for the Parathyroid Extract used in these experiments.

[2] The method is described by Tweedy, W. R., and Koch, F. C., *J. Lab. and Clin. Med.*, 1929, **14**, 747.

[3] McJunkin, F. A., Tweedy, W. R., and Mednicky, W. J., *Arch. Path.*, 1934, **18**, 626.

15 mg. %) produced in a nephrectomized animal. Therefore it appears that the physiological changes which occur as a result of nephrectomy interfere with the mobilization of calcium into the blood.

9001 C

Further Evidence on Hormonal Basis of "Heat" Behavior.*

JOSEPHINE BALL. (Introduced by Carl G. Hartman.)

From the Henry Phipps Psychiatric Clinic and the Carnegie Laboratory of Embryology, Johns Hopkins Medical School.

In an exploratory experiment it was found that doses of 10 to 100 R.U. of estrin (Progynon-B Schering)† failed to have the slightest effect on the behavior of 3 hypophysectomized female rats, although similar amounts consistently brought castrated females into heat. This suggested that estrin produced heat behavior by way of the pituitary and led to a series of experiments which, while somewhat inconclusive, are, nevertheless, of interest because they seem to show that progestin is not the immediate heat behavior hormone in the rat as Dempsey, Hertz and Young[1] believe is the case in the guinea pig.

The same 7 castrated female rats were used in each experiment. The method of measuring varying degrees of sexual excitability has been described elsewhere.[2]

Injection of gonadotropic hormone (Prephysin Chappell)† in doses of 0.04 to 0.80 cc. had no effect, nor did luteinizing hormone† in doses ranging from 5 mg. given in a single injection up to 60 mg. given in 5 increasing doses over a 3-day period. However, 6 mg. of LH raised the sexual excitability of 2 unoperated females that happened to be spontaneously in a condition like that described as "constant estrus" by Witschi and Pfeiffer.[3] The latent period for

* This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

† Grateful acknowledgment is made to the Schering Corporation for the Progynon B and Proluton used in these experiments; to Chappel Bros. for Prephysin; and to Dr. H. L. Fevold for luteinizing hormone.

[1] Dempsey, E. W., Hertz, R., and Young, W. C., *Am. J. Physiol.*, 1936, **116**, 201.

[2] Ball, J., *Comp. Psychol. Monogr.*, in press.

[3] Witschi, E., and Pfeiffer, C. A., *Anat. Rec.*, 1935, **64**, 85.

this reaction was about 4 hours, suggesting a much more direct action than that of estrin which has a latent period of 24 to 48 hours.

These experiments pointed to the need of estrin as a preparatory agent for LH, although some doubt was thrown on such a requirement by the fact that high degrees of heat behavior were exhibited without any cornified cells in the smears of unoperated rats receiving 0.05 cc. Prephysin Chappel daily over long periods of time. Ten such heat periods were observed in 4 rats, indicating that if estrin is essential, subliminal amounts suffice.

An experiment was accordingly arranged which was designed to imitate the behavior picture shown by the "constant estrus" animals treated with LH by keeping castrated females at a low level of receptivity by means of low daily doses of estrin and then injecting LH into these animals in addition to this uniform estrin dosage. Daily doses of 1 to 10 R.U. of estrin were found to keep the animals at the desired low degrees of receptivity, although, interestingly, not always producing cornified smears. LH was then administered in doses varying from 10 mg. in 1 injection to 60 mg. given in 5 injections at hourly intervals. Following the LH injections heat behavior was elicited with slighter stimulation in 11 out of 15 cases. In one case (10 mg.) there was definitely no effect. The other 3 cases (40 and 50 mg.) were doubtful.

These results pointed to a definite effect of LH on estrinized castrated rats. But the behavior so produced was not perfectly typical heat behavior. There is no question about the fact that most of the reactions typical of estrin were more easily elicited. Ear vibration, continuous tense crouching and ready lordosis appeared upon slighter stimulation than before the LH had been injected. But there was almost no darting about the cage after the first quick run to a corner. Moreover, turning of the activity drums in which the animals lived was usually somewhat less after LH injections also, whereas it is well known that the typical heat period is characterized by more rather than less general activity. The animals were also more sensitive to pain, objecting violently to the prick of the hypodermic needle which at other times they gave no sign of feeling. This hypersensitivity and decreased general activity suggest a possible toxic effect of some other ingredient in the extract. However, 3 of the 7 animals showed the same immotility in sex tests when they were later brought to the same degrees of sexual excitability with estrin alone. Apparently the fact that they had been castrated for 7 months at the time of this experiment may have been responsible for some of the immotility but not all of it.

While the animals were still on the daily estrin regime, progestin

was tried to see if it would increase the effect of the estrin. Each rat was injected 2 or 3 times with Proluton Schering, in amounts varying from 0.01 to 0.10 I.U., either in a single injection or in 4 injections given in one afternoon. In no case was there either increase or decrease in the estrin-maintained behavior level. The highest dose used is one-half the effective dose for the guinea pig,[1] an animal about 3 times the size of the rat.

Obviously the experiments with LH will have to be repeated with variations to determine whether it produces a truly specific effect on sex behavior. The failure to obtain any effect from progestin in the rat is, perhaps, open to the criticism that this species may be relatively insensitive to this hormone as regards behavior. Nevertheless, the observations reported bring the endocrinology of heat behavior in the rat, so far as it is known, into line with what it is reasonable to expect from the physiology and time relations of the ovarian and behavioral events occurring at estrus.

9002 C

An Attempt to Induce Nephrotoxins and Experimental Glomerulonephritis by Injections of Homologous Renal Tissue.

ARTHUR E. PARKS, CLAYTON B. ETHRIDGE AND BARRETT L. TAUSSIG. (Introduced by H. A. Christian.)

From the Medical Clinic, Peter Bent Brigham Hospital, and the Laboratory of the Department of Medicine, Harvard Medical School, Boston, Mass.

The rôle of allergy and nephrotoxins in the production of glomerulonephritis has been studied extensively by many investigators. The results of these studies may be found in excellent reviews of the literature by Pearce,[1] Leiter,[2] Masugi,[3] and Fishberg.[4] Recently Masugi has shown that if the blood serum of ducks that have received repeated injections of rabbit kidney be injected intravenously into rabbits, a glomerulonephritis results. Schwentker and Rivers[5] have demonstrated, further, that by autolyzing rabbit brain it is possible to render it antigenic to rabbits so that its repeated in-

[1] Pearce, R. M., *Arch. Int. Med.*, 1910, **5**, 113.

[2] Leiter, L., *Arch. Int. Med.*, 1924, **33**, 611.

[3] Masugi, M., *Ziegler's Beitr.*, 1934, **92**, 429.

[4] Fishberg, A. M., *Hypertension and Nephritis*, Philadelphia, Lea and Febiger, Ed. 3, 1934.

[5] Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.

jection leads to the production of complement fixing antibodies. Consideration of these findings led us to believe that by autolyzing rabbit kidney, it might be made antigenic to rabbits, and that repeated injections might lead to the production of an experimental glomerulonephritis.

Sixteen male rabbits were injected intraperitoneally with emulsions of autolyzed rabbit kidney. Eight of these rabbits were injected with emulsions made from the kidneys of sacrificed rabbits, whereas the remaining 8 were injected with emulsions made from one of their own kidneys that had been removed by unilateral nephrectomy. Thus it was attempted to study the production of "isonephrotoxins" by the injection of modified renal tissue from the same species of animal, and the production of "auto-nephrotoxins" by the injection of modified renal tissue from the same animal.

Strict aseptic precautions were observed in preparing and handling the 16 emulsions used for the injections. Each emulsion represented the tissue of an entire kidney obtained by operation. No attempt was made to wash the blood from these organs for fear that the additional handling might promote bacterial contamination. Following removal, the kidney was held in boiling water for about 3 seconds and was then placed in a sterile stoppered bottle, where autolysis *in toto* was allowed to proceed. Smears stained by the Gram method were made from the interior of these organs at various stages in their autolysis and were examined microscopically for evidence of contamination. Preparations showing more than slight contamination were discarded and new ones substituted. When the desired degree of autolysis had been reached, each kidney was cut up finely with scissors, triturated in a mortar and emulsified by mixing with 35 cc. of sterile normal saline solution.

It was thought that the degree of autolysis of the renal tissue might influence the antigenic properties of the emulsions, and for this reason emulsions were prepared from kidneys autolyzed to different degrees. Those from the injection of Rabbits Nos. 1 to 4 were made from kidneys completely autolyzed by immersion in a water bath for 6 days; of Rabbits Nos. 5 to 8 from kidneys incompletely autolyzed at room temperature for 5 days; of Rabbits Nos. 9 to 12, from kidneys allowed to stand for 14 days; and those for the injection of Rabbits Nos. 13 to 16, from kidneys kept at room temperature for 21 days.

The appropriate emulsion was injected intraperitoneally into each rabbit in doses of 6 to 7 cc., on 4 different occasions, 5 days apart.

The emulsions employed for injecting Rabbits Nos. 7, 10, and 16 were made from kidneys showing slight bacterial or fungus contamination, but the number of organisms seen on repeated smear examinations were so few that rejection was not considered necessary.

The blood sera of the injected rabbits were tested repeatedly by means of the complement fixation reaction to detect the presence of antibodies to the emulsions of autolyzed renal tissue by a modification of a standardized method for performing the Wassermann reaction described by Hinton.[6] The antigen employed in each test was a freshly prepared 1:20 or 1:40 dilution (without filtering or centrifugalization) of the emulsion used for the injections. The blood sera from the normal control and the injected rabbits was obtained by bleeding from an ear vein and was inactivated before use by heating in a water bath at 57°C. for 30 minutes.

The complement was titrated prior to the actual complement-fixation test on each test day, to determine the smallest quantity of complement which caused hemolysis of 0.5 cc. of sensitized sheep erythrocytes (a mixture of equal parts of standardized sheep red blood cells and dilute anti-sheep cell amboceptor). Twice this amount of complement (or 2 units) was used in the subsequent procedures.

The antigens were titrated prior to every complement-fixation test to determine the largest quantity of each antigen which, in the presence of 2 units of complement and 0.5 cc. of sensitized sheep cells caused no inhibition of hemolysis. This and fractional amounts of each antigen were used in the tests themselves. Controls were employed to check against the possibility that inhibition of hemolysis in the test system was due to some factor other than the complement-fixing ability of the treated rabbit serum used. Normal saline was used in all tubes to dilute to constant volume, and 0.5 cc. of sensitized sheep cells was added to each tube after incubation.

Simple urinalysis and determination of the phenolsulphonephthalein excretion were carried out occasionally on each treated rabbit, both before and after the injection of the emulsions, in order to detect any signs of renal damage resulting from the injections.

Results—Pathological Studies. Of the 16 rabbits, 4 died spontaneously at intervals varying from 2 to 66 days after their last injection of emulsion. Each of the 4 displayed symptoms of weakness, loss of weight, diarrhea and collapse. Though only one of these rabbits (No. 16) was injected with a visibly contaminated

[6] Hinton, W. A., *Am. J. Syphilis*, 1920, **4**, 598.

emulsion (fungus), all 4 showed a variable degree of localized or diffuse inflammation of the peritoneum. The remaining 12 showed no evidence of disease during the course of the experiments, and were in good condition when killed. Except for the 4 instances of peritonitis and hypertrophy of a remaining kidney, the autopsies revealed nothing remarkable. Previous operative wounds in the nephrectomized animals were entirely healed. In all animals the organs other than the kidneys were normal in the gross. None of the kidneys from the 16 animals showed any evidence of diffuse nephritis or other abnormalities on gross inspection. Microscopic examination of the kidney sections revealed, in almost every case, a variable degree of interstitial nephritis, characterized by local infiltrations of round cells and focal areas of dilated tubules lined with flattened epithelium. In no instance were there diffuse proliferative or exudative changes in the glomeruli, or any evidence of vascular damage.

Complement-fixation Tests. All sera obtained prior to the injection of the emulsions showed negative reactions. Among the sera obtained 10 days or more after the last injection of the emulsions, those from 4 animals (Rabbits Nos. 1, 4, 5, and 8) showed definite fixation of complement in low titer, on one or more occasions. Such positive results, however, were obtained in only one out of 5 tests for Rabbit No. 1, one out of 5 tests for Rabbit No. 4, one out of 7 tests for Rabbit No. 5, and 2 out of 4 tests for Rabbit No. 8. In every instance later tests on the same animals gave negative results. Altogether out of a total of 50 complement-fixation tests made on the sera of 14 treated rabbits, after the last injection of emulsion, positive fixation of complement was observed for only 4 animals in but 5 instances, and always in low titer.

Renal Function Tests. The urinalysis of the treated rabbits, both before and after the injection of the emulsions, showed transient albuminuria and occasional epithelial cells and casts in sediment. Such abnormalities, however, never were permanent. Later tests showed entirely normal urines from each animal.

The tests for phenolsulphonephthalein excretion made on each rabbit prior to the first injection of the emulsions showed an excretion of 60% or more of the dye in the 2-hour period, except in 2 instances where the excretion was only 24% (Rabbits Nos. 3 and 15). On tests made after the last injection of the emulsions each animal showed excretions of the dye closely similar to its previous result.

Conclusion. An attempt to induce in rabbits complement-fixing

antibodies and an experimental glomerulonephritis by injections of autolyzed homologous kidney was essentially negative in its results.

9003 C

Stability of Vitamin C, and Absence of Ascorbic Acid Oxidase in Citrous Fruits and Milk.

HENRY TAUBER.
Long Island City, N. Y.

It has been shown recently that in certain plants such as Hubbard Squash and Summer Squash, a powerful enzyme is present which oxidizes rapidly vitamin C[1, 2] although these plants contain almost none of this vitamin. Similar is the case with cucumbers. Statements have appeared more recently that "plant tissues which contain ascorbic acid apparently also contain an ascorbic acid oxidizing enzyme," and that the partial destruction of vitamin C in cow's milk is also brought about by ascorbic acid oxidase. The present writer could not find ascorbic acid oxidase in mammalian tissue,[3] and Roe and Barnum[4] found in human and rat blood cells and plasma an enzyme which reduces the reversibly oxidized form of ascorbic acid, thus having just the opposite effect from the ascorbic acid oxidase of plant tissues.

The aim of the present work was to find out whether the vitamin C content of juices of citrous fruits, which are excellent sources of the vitamin, is exposed to the destructive action of the ascorbic acid oxidase. In other words, whether these juices contain the oxidase.

Table I shows that the vitamin C content of the juices of oranges,

TABLE I.
Analysis of Vitamin C per cc. of Fruit Juice.

	After 5 hr. at 6° mg.	After 5 hr. at 38° mg.
Orange Juice (type *t*)	.51	.46
Lemon " (" *t*)	.56	.53
Tangerine " (" *a*)	.39	.33
Grapefruit " (" *a*)	.46	.44

[1] Tauber, H., and Kleiner, I. S., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 577.
[2] Tauber, H., Kleiner, I. S., and Mishkind, D., *J. Biol. Chem.*, 1935, **110**, 211.
[3] Tauber, H., *Experimental Enzyme Chemistry*, Burgess Pub. Co., Minneapolis, 1936.
[4] Roe, J. H., and Barnum, G. L., *J. Nutrition*, 1936, **11**, 359.

tangerines, lemons and grapefruits is not much affected when kept for 5 hours at 38° as compared to control samples which were placed in a refrigerator at 6° for the same amount of time. The slight decrease in reducing power does not necessarily indicate an irreversible oxidation. The results show, however, that there is no ascorbic oxidase in these fruit juices and that the vitamin keeps fairly well even at 38°. It is well known that the pH of these fruit juices is a stabilizing factor of vitamin C. Samples of orange juice which have been adjusted to pH 6.5 with ammonium hydroxide or $CaCO_3$, however, kept equally well and no evidence of enzyme action could be noticed at this pH either. 2,6-Dichlorobenzenone indophenol was used for the titration of vitamin C. It should be noted that oranges and tangerines contain a small amount of a substance other than vitamin C, which reduces the oxidation-reduction indicator.[5]

Since it was recently suggested that milk contains ascorbic acid oxidase[6] thus causing partial destruction of vitamin C, this point was also tested. To a 100 cc. sample of fresh raw milk, 25 mg. of ascorbic acid, which was dissolved in 10 cc. of distilled water, was added* and kept for 3 hours at 38°. To a second sample of 100 cc. of milk the same amount of ascorbic acid was added just before titration (zero time). Only about 3% of the added vitamin C was oxidized within 3 hours, indicating that at most there is only a trace of ascorbic acid oxidase in cow's milk. The pH of the milk was 7.0. The vitamin C was determined by titration, after coagulation of the milk proteins by adding 2 cc. 2/3 N H_2SO_4 to 20 cc. of milk, without filtration, so as to eliminate the error owing to slow filtration, as proposed by Sharp.[6] The partial destruction of vitamin C in cow's milk is probably due to traces of copper in the milk introduced by feeding or by technical means, but not to enzyme action.

[5] Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, 1935, **110**, 559.

[6] Sharp, P. F., *Science*, 1936, **84**, 461.

* Ascorbic acid was added because milk is a very poor source of vitamin C. The samples analyzed contained 17.5 mg. of vitamin C per 1000 cc. of milk.

9004 P

Factors Affecting Human Potassium Tolerance.

RAYMOND L. ZWEMER AND RICHARD TRUSZKOWSKI.

From the Department of Anatomy, College of Physicians and Surgeons, Columbia University.

A relation of the adrenal cortex to potassium metabolism is substantiated by the following facts. In experimental adrenal insufficiency,[1, 4] and in Addison's disease[3, 6] there is a definite rise in plasma potassium. This high potassium can be lowered by injection of adrenal cortex extract.[2, 6] In adrenal insufficiency there is an increased susceptibility to exogenous potassium,[7, 8, 9] which has been quantitatively contrasted with normal potassium tolerance in experimental animals.[10] The application of potassium tolerance determinations in the human is here briefly described.

Solutions of potassium salts were administered by mouth, the dose being 10 mg. or 20 mg. of K per pound of body weight. Duplicate determinations of potassium were done for plasma and whole blood taken from the finger or ear immediately before taking the drink, and at intervals of 30 min., 1 hr., and 2 hr., thereafter. Simultaneous hematocrit readings enabled us to calculate the red cell potassium content. If similar amounts of potassium contained in food are taken, slower assimilation makes it necessary to continue the test for 5 hours.

Seventeen human potassium tolerance curves on 12 individuals have been completed to date. Since this preliminary paper does not permit detailed discussion of all the curves, a few typical ones only are shown.

The plasma K of normal individuals is not affected by ingestion of 10 mg. K per pound body weight (Graph 1). In Addison's disease, however, there is a rapid and very considerable rise in plasma K (Graph 2), much greater than can be accounted for by

1 Bauman and Kurland, *J. Biol. Chem.*, 1926, **71**, 281.

2 Zwemer and Sullivan, *Endocrinology*, 1934, **18**, 97.

3 Urechia, Benetato and Retzeanu, *Compt. Rend. Soc. Biol.*, Paris, **119**, 439.

4 Truszkowski and Zwemer, *Biochem. J.*, 1936, **30**, 1345.

5 Marañon, Cellazo, Barbudo and Torres, *Arch. Med. Cir.*, 1934, **37**, 893.

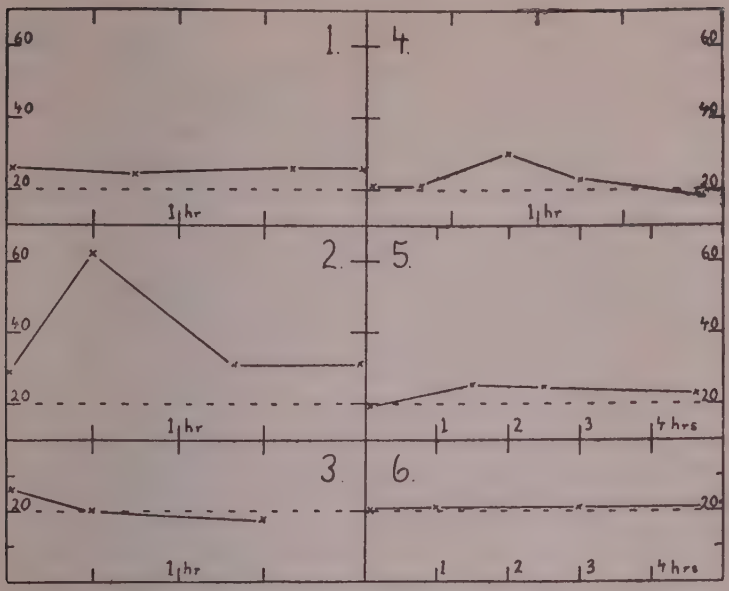
6 Allot, *Lancet*, 1936, **230**, 1406.

7 Zwemer and Truszkowski, *Science*, 1936, **83**, 558.

8 Allers, Nilson and Kendall, *Proc. Staff Meet., Mayo Clinic*, 1936, **11**, 283.

9 Wilder, Snell, Kepler, Rynearson, Adams and Kendall, *Proc. Staff Meet., Mayo Clinic*, 1936, **11**, 273.

10 Zwemer and Truszkowski, *Endocrinology*, 1937, **21** (Jan.), (in press).



EXPLANATION OF GRAPHS.

The mean of duplicate plasma potassium determinations is given in mg. per 100 ml. of plasma. Time is given in hours.

Graph 1. Plasma K of normal person remained unchanged after 10 mg. of potassium per pound body weight given by mouth as a solution of potassium salts.

Graph 2. Rise in plasma K of an Addisonian after a dose identical with that given above.

Graph 3. The 10 mg. per pound body weight dose given to an individual subject to attacks of petit mal did not increase plasma K.

Graph 4. A normal person had a transient rise in plasma K when given 20 mg. K per pound.

Graph 5. Eating of food containing enough K to give an approximate intake of 20 mg. K per pound body weight was followed by an increase in the plasma K of an individual with suspected adrenal insufficiency.

Graph 6. A similar meal to the same individual after treatment.

hemoconcentration. At the end of 2 hours both cell volume and plasma K are again approaching the initial values, but the red cell K content has increased, the increase being perhaps compensatory. Another graph illustrating the 10 mg. dose is from a patient subject to attacks of petit mal (Graph 3). This showed the peculiar phenomenon of a drop in plasma K with a rise in the red cell K content. The constancy of the hematocrit determinations would suggest that the red cell K rise was not due to a shift of fluid between plasma and cells.

Twenty mg. of K per pound body weight gives a definite rise in plasma K in normal individuals (Graph 4). In a few cases in which the test was repeated on the same individual, the character of the

response appeared to be constant. In certain allergic conditions the initial plasma K was found to be higher than normal and the return to the initial level after the 20 mg. dose was somewhat delayed. The relation of the adrenal cortex to allergy has been discussed elsewhere,[11] and in view of its general application we are continuing these studies, in cooperation with the Department of Dermatology.

Although the plasma K of normal individuals does not appear to rise after an average meal, a patient with suspected adrenal insufficiency showed a definite rise in plasma K following a test meal consisting of steak, french fried potatoes and raw cabbage (Graph 5). These were chosen because of their relatively high K content. After a week's rest and treatment with adrenal cortex extract the patient was improved subjectively and a similar test meal produced no rise in plasma K (Graph 6). In contrast to the previous experiment, red cell K fell during the digestion period.

The 5-hour feeding experiments are less satisfactory than the administration of a K drink, both because of the time necessary to complete the test, and of the uncertain K content of the food. They might, however, be used for preliminary tests in certain cases. We feel that either test would be safer and quicker than sodium chloride deprivation as described by Loeb.[12] Any untoward effect can immediately be treated by injection of adrenal cortex extract, which we have found to lower blood K within 15 minutes of injection.

In conclusion, we suggest that the K tolerance test as outlined be used in the diagnosis of corticoadrenal insufficiency.

---

11 Wolfram and Zwemer, *J. Exp. Med.*, 1935, **61**, 9.

12 Loeb, *J. A. M. A.*, 1935, **104**, 2177.

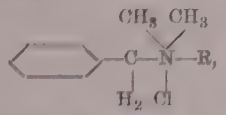
9005 P

A Mixture of High Molecular Alkyl-dimethyl-benzyl-ammonium Chlorides as an Antiseptic.*

CECIL G. DUNN. (Introduced by A. E. Meyer.)

From the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

Recent investigations have shown that certain alkyl-dimethyl-benzyl ammonium chlorides,



possess characteristics highly desirable in an antiseptic or germicide. The product is obtained as a mixture of homologues in which the alkyls represent radicals derived from the fatty acids of coconut oil. The mixture has been found to be quite uniform in composition.

This compound is an amber-colored solid of soap-like consistency. From the solid is generally prepared a 10% aqueous stock solution, which is slightly acid in reaction and which has a surface tension of less than 36 dynes/cm.

Germicidal tests were carried out according to F.D.A. methods.† The cultures of *Staphylococcus aureus* and *Eberthella typhosa* used were secured from the Food and Drug Administration at Washington, D. C., the streptococci from the Boston City Hospital, and the other organisms from the collection of the Department of Biology and Public Health at the Massachusetts Institute of Technology.

All the bacteria were cultivated in Reddish broth, with the exception of the Streptococci, which were grown in Bacto-brain-heart infusion containing 0.1% agar. The molds were grown on Sabouraud's dextrose-agar and subcultured in Sabouraud's dextrose-broth.

Table I shows the results of some germicidal tests.

Both Gram-positive and Gram-negative pathogenic organisms were readily destroyed by the ammonium compound in high dilution. The hemolytic streptococcus, *E. typhosa* and *C. hominis* were

* Contribution No. 82 of the Department of Biology and Public Health, Massachusetts Institute of Technology. Aided by a grant from the Alba Pharmaceutical Company.

† Food and Drug Administration Methods of Testing Disinfectants and Antiseptics. Circular No. 198. U.S.D.A. 1931

destroyed in the highest dilution at 37°C. *Monilia albicans* was most resistant at 20°C., but was quickly destroyed by a 1/10,000 dilution.

TABLE I.

Organisms	Average phenol-coefficients		Highest dilution of the chemical destroying the organism in 10 but not 5 mins. (Average values)	
	20°C	37°C	20°C	37°C
S. aureus	279	407	1/20,000	1/35,000
Eberthella typhosa	250	429	1/20,000	1/70,000
E. coli	160	358	1/12,000	1/40,000
Str. hemolyticus	435	579	1/40,000	1/95,000
Str. viridans	384	434	1/35,000	1/65,000
Cryptococcus hominis	214	395	1/24,000	1/70,000
Monilia albicans	111	274	1/10,000	1/35,000

Effect of Temperature. The germicidal action of the chemical against *Staphylococcus aureus* and *E. coli* at low temperatures was determined. At lower than 1°C., *S. aureus* was destroyed in 10 but not in 5 minutes by a dilution of 1/4,500 (average value), and *E. coli* by a dilution of 1/1,000. Neither freezing nor storage at above 50°C. for a period of 18 days, caused any apparent reduction in the germicidal action of the chemical.

Action in the Presence of Organic Matter. Tests were carried out in which normal horse serum was substituted for some of the dilution water in preparing dilutions of the chemical. All the bacteria listed in Table I were readily destroyed in the presence of 20% serum—twice the amount advocated by F.D.A. Methods—at both 20° and 37°C. *E. coli* was the most resistant micro-organism but it did not survive for 10 minutes in a 1/3,200 dilution at 37°C.

In the presence of 50% serum, a 1/200 dilution of the chemical destroyed *Staphylococcus aureus* in less than 15 seconds at 37°C., and in less than 30 seconds at 20°C. A dilution of 1/1,200 was effective in destroying this organism in 10 but not 5 minutes in the presence of 50% serum at 20°C. In comparison, a well known commercial mercurial—a 1/200 tincture—failed to destroy *S. aureus* at 20°C. when diluted to 1/400 by the presence of 50% serum.

Bacteriostatic Tests. The tests were run by incubating tubes at 37°C., each of which contained 8 cc. of Reddish broth, 1 cc. of a dilution of the chemical and 1 cc. of a 1/10 aqueous dilution of the test organism. Alkyl-dimethyl-benzyl ammonium chlorides pre-

vented the growth of the Gram-positive bacteria, *S. aureus* and *B. subtilis*, in a dilution of 1/100,000. Under similar conditions a 1/20,000 dilution of the chemical prevented the growth of *E. coli*.

The mixture of alkyl-dimethyl-benzyl ammonium chlorides investigated possesses high bactericidal efficacy, and has compared favorably with some of the best antiseptics in use.

9006 C

Integrity of the Skin in Relation to Cutaneous Absorption of Insulin.*

MAURICE BRUGER AND JAMES FLEXNER.

From the Department of Medicine, New York Post-Graduate Medical School and Hospital, New York.

Telfer[1] reported that insulin in the form of an ointment when rubbed into the skin of rabbits produced a fall in blood sugar. This work stimulated further investigations along similar lines and several reports from a number of workers have appeared in the literature; however, the results on insulin inunction have by no means been uniform.

Of particular interest is a recent report by Major[2] who showed that a solution of insulin mixed with diethylene glycol monoethyl ether when rubbed into the skin of rabbits produced a marked fall in the blood sugar. The experimental procedure adopted by Major was as follows: the skin over the abdomen was shaved and several drops of glycerine first rubbed in over the shaved area. Ten to 15 minutes later, 25 units of insulin was applied. The blood sugar diminished markedly, and in a number of animals hypoglycemic convulsions ensued 3 to 4 hours later. Major further observed that in the course of repeated experiments on the same rabbits, some animals apparently became refractory after the insulin had been applied to the skin of the abdomen 3 or 4 times. He further stated that such animals were found not to be refractory if the hair was shaved off the back or thigh and the insulin applied on this fresh area.

Our frankly negative results on insulin inunction in humans (unreported studies) led us to believe that the positive results obtained

* Aided by a grant from the Harriet Weil Fund.

1 Telfer, S. V., *Brit. Med. J.*, 1923, **1**, 715.

2 Major, R. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 775.

by Major may have depended in good measure, if not entirely, on the degree of the integrity of the skin. In other words, we felt that the application of insulin to an abraded (shaved) skin would permit the absorption of insulin; on the other hand, insulin applied to a depilated area several days after the shaving was carried out would not be absorbed to any appreciable extent since the previously abraded area had been given time to heal. Thus, we felt that true refractoriness of the skin to the dermal application of insulin, as contended by Major, may not actually exist.

Tables I, II and III prove our contention. In those rabbits in which a small area of the skin was first shaved and healing of the abrasions permitted for 5 to 7 days, the application of 50 units of insulin in diethylene glycol monoethyl ether failed to produce any effect on the blood sugar (Table I). Again, in those rabbits in

TABLE I.

Exp. No.	Rabbit No.	Area shaved	Days between shaving and insulin application	Time	Blood sugar, mg. %	Remarks
1	1	right back	5	10:22	124	
				10:33-10:38		glycerine applied
				10:38-10:42		50 units insulin
				11:12	122	
				11:42	115	
				12:42	123	
				1:42	132	
				2:42	125	
2	2	left back	6	11:17	114	
				11:38-11:43		glycerine applied
				11:43-11:48		50 units insulin
				12:18	121	
				12:48	126	
				1:48	152	
				2:48	150	
				3:48	150	
3	3	abdomen	5	10:10	96	
				10:18-10:23		glycerine applied
				10:23-10:28		50 units insulin
				10:58	144	
				11:28	125	
				12:28	132	
				1:28	134	
				2:28	125	
4	3	left back	7	10:39	139	
				10:44-10:49		glycerine applied
				10:49-10:54		50 units insulin
				11:24	138	
				11:54	133	
				12:54	120	
				1:54	112	
				2:54	115	

TABLE II.

Exp. No.	Rabbit No.	Area shaved	Minutes between shaving and insulin application	Time	Blood sugar, mg. %	Remarks
5	1	left back	15	11:14	99	
				11:28-11:33		glycerine applied
				11:33-11:38		50 units insulin
				12:08	66	
				12:38	53	weak, crawling
				1:38	36	convulsions, opisthotonus and death.
6	2	right back	15	10:28	109	
				10:43-10:48		glycerine applied
				10:48-10:53		50 units insulin
				11:23	74	
				11:53	66	
				12:53	52	
				1:53	46	slight tremors
				2:53	76	
7	4	left back	20	10:42	119	
				10:55-11:00		glycerine applied
				11:00-11:05		50 units insulin
				11:35	93	
				12:05	69	
				1:05	70	
				2:05	72	
				3:05	86	

TABLE III.

Exp. No.	Rabbit No.	Area shaved	Interim between shaving and insulin application	Time	Blood sugar, mg. %	Remarks
8	3	abdomen	7 days	10:15	130	
				10:32-10:37		glycerine applied
				10:37-10:42		50 units insulin
				11:12	188	
				11:42	183	
				12:42	130	
				1:42	120	
				2:42	143	
9	3	abdomen reshaved	15 min.	10:44	146	
				10:55-11:00		glycerine applied
				11:00-11:05		50 units insulin
				11:35	115	
				12:05	57	
				1:05	31	Convulsions. 8 cc. 50% glucose intravenously.
				2:40	73	

which an area of the skin was shaved and the insulin applied 15 to 20 minutes later, a distinct fall in the blood sugar occurred (Table

II). Of interest are the experiments on Rabbit 3 in Table III. On October 20 an area over the abdomen was shaved. Seven days later the application of 50 units of insulin to this area failed to decrease the blood sugar (in fact, there was a temporary rise in the blood sugar probably due to the excitement manifested by this animal). On the following day, this same abdominal area, upon which the previous application of insulin failed to produce any effect, was reshaved and 50 units of insulin rubbed in approximately 15 minutes later. There was a marked fall in the blood sugar requiring the intravenous administration of dextrose to overcome the hypoglycemic convulsions.

Conclusions. The absorption of insulin by the skin of rabbits is dependent upon the integrity of the integument. The intact skin shows little or no absorption, whereas a recently abraded skin, such as produced by shaving, permits the absorption of an appreciable amount of insulin.

We are indebted to the Eli Lilly Company for a generous supply of powdered insulin.

9007 C

Fluctuations in Type 2 Pneumococcus Antibody During the Menstrual Cycle.*

VICTOR ROSS AND LENORE R. PEIZER.

From the Bureau of Laboratories, Department of Health, New York City.

In the course of measuring the duration of the protective power of the sera of a number of persons who had been fed pneumococcal vaccine, fluctuating values for Type 2 were observed in the blood of 2 women. In the case of A.E.T., No. 10,† 1 cc. serum from blood drawn at 2 different times early in the investigation protected mice against approximately 500 fatal doses. Seven and a half months later the serum protected against only minimal numbers of pneumococci (*ca.* 5 fatal doses). However, after a lapse of 27½ months more the blood again protected well (*ca.* 5000 fatal doses per cc. See Table, 1/28/35). The other person's (L.P., No. 18†) blood possessed no protective power when first examined, then acquired

* This work has been made possible by a grant from Lederle Laboratories, Inc., Pearl River, N. Y., to whom the writers wish to express their thanks.

† The initials and the numbers are those used in the report, "Oral Immunization of Humans against the Pneumococcus." *J. Immunol.*, 1934, **27**, 307.

a small amount (*ca.* 50 fatal doses per cc.) which was lost after 12 months. Thirty-three months after the first determination the blood contained a considerable amount of antibody (*ca.* 5000 fatal doses per cc.). The fact that this sample of blood had been collected one day before menses began (January 14. See Table) suggested that the observed rise and fall in protective antibody for Type 2 pneumococcus might be related to the menstrual cycle. Inquiry revealed that the most recent sample of blood from A.E.T. which protected had been taken on the 8th day of the cycle. Two months later the first of 4 additional tests, done at different stages of the menstrual cycle over a period of 2 months, was carried out. Fluctuating concentrations were found in the serum of both individuals.

Others have reported that the bactericidal power of blood for a variety of organisms is related to the stage of the menstrual cycle. Geller and Sommer[1] reported a drop in bactericidal power of whole blood for hemolytic streptococci just before and during the first part of the menses followed by a rise during the latter part, or soon after. Dresel and Keller[2] reported that serum was bactericidal for anthrax bacilli during menstruation but not at other times. These authors considered this property to be non-specific in the immunological sense. Molinengo[3] found that the destructive power of blood for several bactericidal species derived from the vagina sinks in the premenstrual period and rises immediately following the end of menses.

Jungeblut and Engle[4] found that one individual's serum neutralized poliomyelitic virus on the first day of menses, but failed to do so on the 15th day of the menstrual cycle, and that another person's serum neutralized on the 15th day but not on the first day. They suggest that the power to inactivate the virus may change in accordance with fluctuations in endocrinal balance. Aycock[5] has recently reported that 4 out of 5 castrated female monkeys developed poliomyelitis following intranasal instillation of the virus while only one out of 5 similarly treated animals which received injections of estrin prior to the viral instillation succumbed. Söda,[6] however, reported no difference in the formation of normal agglutinins and bacteriolysins, nor immune agglutinins and bacteriolysins for typhoid bacilli in control and ovariectomized rabbits.

---

1 Geller, F. C., and Sommer, W., *Arch. f. Gynak.*, 1927, **31**, 293.

2 Dresel, E. G., and Keller, W., *Z. Hyg.*, 1922, **97**, 151.

3 Molinengo, L., *Gior. di Batt. e. Imm.*, 1934, **13**, 379.

4 Jungeblut, C. W., and Engle, E. T., *J. Immunol.*, 1933, **24**, 267.

5 Aycock, W. Lloyd, PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 573.

6 Soda, G., *Tarivan Igakkai Zasshi*, No. 269, August, 1927. (Summary in German.)

TABLE I.

Protective Antibody Against Type 2 Pneumococcus at Different Stages of the Menstrual Cycle.

	Subject L. P., No. 18										Subject A. E. T., No. 10									
Dose cc.	1 day before menses 1/14/35		12th day of cycle 3/20/35		2d day of menses 5/6/35		20th day of cycle 5/25/35		9th day of cycle 6/8/35		8th day of cycle 1/28/35		14th day of cycle 3/29/35		1st day of menses 5/8/35		21st day of cycle 5/29/35		9th day of cycle 6/8/35	
	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.	Con.	Ser.
10–9	2.3	S.S.	S.1		S.2	S.S.	S.1	S.S.	S.S.	S.S.	S.2	S.S.	S.1		S.2	S.S.	S.1	S.S.	S.S.	S.S.
10–8	1.1	S.S.	1.1	2.2	1.1	S.S.	1.1	S.S.	1.1	S.S.	1.1	S.S.	1.1	1.2	2.1	S.S.	2.1	S.S.	1.1	S.S.
10–7	1.1	S.S.	2.2	S.1	1.1	S.S.	1.1	S.S.	1.1	S.S.	1.1	S.S.	2.2	S.1	1.1	S.S.	2.1	2.1	1.1	S.S.
10–6	1.1	S.2	1.1	2.3	2.1	S.3	1.1	3.3	1.2	S.1	1.1	S.S.	1.1	2.1	2.1	S.S.	1.1	2.1	1.2	2.2
10–5				1.1		2.2		2.1		2.2				2.2		2.2		2.2		1.1

All intervals in relation to the menstrual cycle were determined by observation except that for A.E.T. on 1/28/35 which was calculated on 3/29/35. June 8, 1935, was the 9th day by observation (A.E.T.); menses started about 7 days sooner than expected. S. = Mouse survived. The numerals give the number of days between the injection and time of death of the mouse. Two mice were injected with each dose of culture. Con. = Control mice. Ser. = Serum treated mice.

In the present experiments, the protective antibody was measured by injecting mice intraäbdominally with a mixture of 0.20 cc. undiluted serum and 0.20 cc. pneumococcal culture diluted to contain the desired dose. The same strain of pneumococcus was used throughout all the experiments recorded here as well as those previously reported.

Blood was collected from L.P. on the 2d, 9th, 12th, and 20th days of the menstrual cycle (not all in the same cycle). (See table and chart). The protective antibody content is high on the second day. It is lower on the 9th day. On the 12th day the serum possesses no protective power. On the 20th day the titre is moderate once more. A similar fluctuation in protective power can be seen to have occurred in the case of A.E.T. The titre on the first day is high. In the 9th-day sample the concentration is considerably lower, and in that of the 14th day there was little or no Type 2 antibody. In the 21st day sample the concentration was about the same as in the blood drawn on the 9th day.

Protective Antibody Against Type 2 Pneumococcus at Different Stages of the Menstrual Cycle.

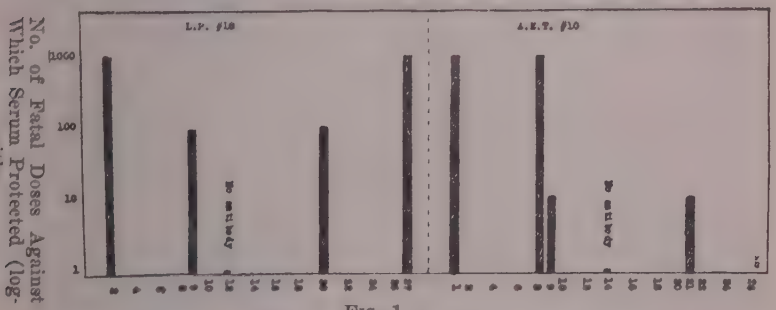


FIG. 1.

Day of Menstrual Cycle.

The vertical bar above the 27th day (L.P.) represents the value for the "day before" shown in the table.

There were fluctuations also in the Type 1 antibody content, but these were less marked than in the case of Type 2. The highest values for L.P.'s serum were obtained on the "day before", on the 2nd day and on the 9th day, that is on the same days when Type 2 values were high. In the case of A.E.T. the highest values for Type 1 were observed on the 1st and 8th days and lower values on the 9th, 14th and 21st days.

This rise and fall in titre suggests a relationship between antibody content and endocrinal factor, at least in some women. It will be observed that the protective power was high in the sera of both

subjects during menstruation and for several days after, and then fell off. It appears to rise again before the onset of the next period. Frank and Salmon[7] have recently reported a maximal concentration of gonadotropic hormone in the blood of normal, menstruating women between the 9th and 12th days. Frank and Goldberger[8] observed maximal amounts of estrogenic hormone in the blood between the 7th day preceding menstruation and the onset of the period, and that there was least immediately after menstruation following which the concentration began to rise. It may be, therefore, that a rise in estrogenic hormone in the blood is accompanied, in some women, by a stimulation of the antibody-producing cells, the resulting increased titre outlasting the increase in hormone. When we injected amniotin‡ simultaneously with Type I pneumococcus into male rabbits there resulted a somewhat greater concentration of agglutinins than in rabbits receiving the vaccine alone, following the first 3 injections of organisms. This difference was not, however, maintained on further treatment.

Is the season of the year causally related to the fluctuations? Graesser and Harrison[9] found that the pneumococcicidal power of the sera of 7 of 34 normal adults showed a drop against Type I between the fall of 1931 and the spring of 1932. Of these 7, two also showed a drop against Type 2. There were no increases in any of the 34. Ward[10] found no change in pneumococcicidal power of whole blood in 9 out of 10 persons when tests were done from time to time. No mention is made of the sex of the subjects by these authors. Orr, MacLeod and Mackie[11] observed, in sheep sera, a seasonal fluctuation in bacteriolytic effect on *B. coli*, and agglutinin for *B. abortus* and *B. paratyphosus* B. Pritchett[12] observed seasonal variations in the mortality of mice following *per os* administration of *B. pestis cavia*. Season could hardly have influenced our data because the last 4 determinations were made within a period of 2 months. Furthermore, since L.P.'s serum had a high concentration of protective antibody on May 6, 1935, and no antibody on May 1, 1933, and A.E.T.'s blood protected well on April 4, 1932, but not at all on

[7] Frank, R. T., and Salmon, N. J., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1287.

[8] Frank, R. T., and Goldberger, M. A., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1663.

‡ Estrus-producing hormone kindly supplied by Dr. John F. Anderson of E. R. Squibb & Sons.

[9] Graesser, J. B., and Harrison, M. C., *J. Exp. Med.*, 1933, **58**, 245.

[10] Ward, K., *J. Exp. Med.*, 1930, **51**, 675.

[11] Orr, J. B., MacLeod, J. J. R., and Mackie, T. J., *Lancet*, 1931, **220**, 1177.

[12] Pritchett, I. W., *J. Exp. Med.*, 1926, **43**, 173.

March 29, 1935, it is unlikely that the fluctuations are related to changes in seasons.

Gaskell[13] reported that the virulence of pneumococci varies with the season. It can be seen from the table that the fatal dose for mice remained fairly constant during the January to June 1931 tests and that high and low values for antibody bore no relation to this dose. Hence a fluctuation in virulence could not have been the cause of the observed differences.

It is necessary to consider a further explanation. Goodner and Horsfall[14] showed that lipids (cholesterol, lecithin, cephalin) are adsorbed by the immune precipitate when antipneumococcal serum and the capsular polysaccharide are mixed. Their experiments indicate that the addition of cholesterol to a mixture of antipneumococcal serum and Type I pneumococci lowers the protective power of horse serum and probably also of rabbit serum. It has been reported by Ikeda[15] that during menstruation the total cholesterol of the blood drops, and that 5 days later the values have not quite reached their normal level. This is the period during which we observed high protective values. Although there is as yet no evidence that cholesterol acts with human serum containing protective antibody as it does with antipneumococcal animal sera, it is necessary to keep these data in mind in attempting to arrive at a more detailed explanation for the present results.

It is of interest to note that several investigators have found that serum-globulin rises sharply during menstruation. This is the constituent with which protective antibody is associated in therapeutic sera.

Summary. The concentration of Type 2 pneumococcal protective antibody in the serum of 2 women fluctuated during the menstrual cycle, being greater just before during and shortly after menses and less after the 12th day of the cycle.

We wish to record our appreciation to Dr. Wm. H. Park, through whose courtesy in providing the necessary facilities we were able to carry out these experiments.

[13] Gaskell, J. F., *J. Path. and Bact.*, 1927, **30**, 515.

[14] Goodner, K., and Horsfall, F. L., *Jr. J. Exp. Med.*, 1934, **60**, 827.

[15] Ikeda, M., *Jap. J. Obst. and Gyn.*, 1932, **15**, 480.

9008 C

Tissue Affinity of Shope Papilloma Virus.

JOHN G. KIDD AND ROBERT J. PARSONS. (Introduced by Peyton Rous.)

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

The virus causing papillomatosis in western cottontail rabbits is capable of producing papillomas in other species of rabbits and hares, but not in guinea pigs, rats, mice, dogs, goats or cats.[1] It causes growths which derive from the surface epidermal cells, not the specialized cells of the hair follicles or sebaceous glands, and direct inoculation of the virus into the lining epithelium of the oral cavity, trachea, rectum, genito-urinary tract, or salivary glands of susceptible rabbits has failed to result in lesions, as has intravenous inoculation, save when the virus localizes in the epidermis.[2] Since these observations were made with only a few virus materials it has seemed desirable to expand them, using virus fluids of known high potency and altering the epithelium to be tested in ways that might render it susceptible. In the course of the work an endemic oral papillomatosis of domestic rabbits was encountered which is described in an accompanying paper. The existence of this disease has added a further interest to the delineation of the tissue affinities of the Shope papilloma virus.

Tests of Normal Epithelium Other Than Skin. A 10% suspension of Shope virus of proven activity was tattooed freely into the under surface of the tongue, the cheek, lips, gums, nares, conjunctiva, genital mucous membrane, and skin of the sides of 4 normal, adult, domestic rabbits. In addition, it was tattooed into the buccal mucous membrane at the base of each lower incisor tooth and outwards along a continuous line to the inner surface of the lower lip and thence across the mucocutaneous junction and onto the skin of the lower lip. Virus was also tattooed into the genital mucous membrane and outwards along unbroken lines onto the adjoining skin. Characteristic papillomas appeared in all the animals after 10 to 14 days, but only in the skin where the virus had been inoculated. The growths enlarged progressively and those of the skin about the mouth and genitalia extended to within 1 mm. of the mucocutaneous junction, but never further. The animals were observed over

[1] Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607; Beard, J. W., and Rous, P., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 191.

[2] Rous, P., and Beard, J. W., *J. Exp. Med.*, 1934, **60**, 701.

the next 8 weeks; the skin growths in all animals continued to enlarge but no papillomas developed elsewhere. The experiment was repeated on 3 normal belted Dutch rabbits, using a different active virus material. All developed papillomas where inoculated on the skin, but no growths appeared at the sites of inoculation into the oral or genital mucous membrane, or into the conjunctiva.

Tests of the Susceptibility of Epithelium Keratinizing as a Result of Avitaminosis A. It is well known that widespread epithelial alterations (squamous metaplasia, keratinization) occur in animals deprived of vitamin A for a sufficient period. It was thought that under such circumstances changed epithelial tissues might prove susceptible to the Shope virus, although refractory to its action in their normal state.

A state of vitamin A deficiency, with weight loss and gross xerophthalmia was brought about in 6 of 7 young Dutch rabbits fed 6 to 12 weeks on a diet* consisting of

Regenerated cellulose	20
Casein	15
Sugar	10
Corn starch	37
Wesson oil	4.0
Yeast	10.0
Minerals[3]	4.0

2 drops of viosterol 250 D was added to the above and the diet was supplemented with white turnips carefully selected and pared of green tops. Two to 12 days after gross xerophthalmia had become evident all the rabbits were inoculated with large doses of active Shope virus. The virus was tattooed into the mouth, nares, conjunctivae, and vagina or penis, and in some instances large amounts were given intravenously and *per os*. Four of the animals survived the inoculations 2 weeks or longer and 2 developed characteristic papillomas on the skin, one as a result of localization out of the blood stream into an injured area, the other as a result of direct contamination. The condition of the rabbits was poor and they died 3 to 8 weeks after the virus inoculations. The diet had been maintained, and the elapsed period after inoculation had exceeded the ordinary incubation period of the virus. At autopsy the changes consequent on the vitamin deficiency were pronounced (gross xerophthalmia, weight loss, microscopic keratinization of the cornea,

* We are indebted to Dr. Alwin M. Pappenheimer and his associates, Dr. Madsen and Dr. Goettsch, for guidance as to the diet, as also for generous gifts of ground cellophane (regenerated cellulose).

[3] Hawk, P. B., and Oser, B. L., *Science*, 1931, **74**, 369.

conjunctiva, buccal mucous membrane and other epithelial tissues), yet nowhere had the virus produced papillomas save on the skin. Here the growths were characteristic.

Virus Injections into the Mammary Gland. In an attempt to infect the mammary epithelium with Shope virus 3 adult, female, gray-brown domestic rabbits were used. One of the rabbits had suckling young, the others had had litters some time previously but their breasts were inactive. Several areas of mammary tissue on each rabbit were "prepared" for virus infection by injecting into them a saturated solution of Scharlach R in olive oil. Three such injections were made into each area at intervals of 3 to 4 days, with result in firm, fleshy, subcutaneous lumps, which others have shown to consist of proliferating mammary epithelium.[4] Five days after the last Scharlach R injection, active Shope virus was inoculated into the under side of these reactive lumps, and also into several breast regions which had not been treated, by means of a hollow needle inserted through a slit in the skin some distance away. In addition, the ducts of several of the glands were distended with virus fluid inoculated through a blunt needle inserted into the nipple. The animals were observed for 5 to 10 weeks. No mammary growths developed, but in 2 of the rabbits papillomas appeared where the virus had come into contact with the epidermis of the nipple.

Tests with Embryo Skin. Previous findings have indicated that embryo rabbit epithelium is insusceptible to the action of the Shope virus. Two additional experiments, in which the mother was used as the test animal, have corroborated this finding.

The 15-day embryos were removed from a domestic rabbit by hysterectomy. Bits of the skin from several areas were taken from all the embryos. These were pooled and hashed fine with knives, under aseptic conditions. Half the minced fragments were then suspended in ordinary Tyrode under the same conditions. Equal portions of both materials were then injected through the slit skin into comparable situations in the fore and hind leg muscles, the axillae, and groins of the doe from which the embryos had been removed. Small nodules, 3 to 5 mm. in diameter, developed in both groins during the next 3 weeks, but later these gradually dwindled in size. At autopsy 10 weeks after the implantations several tiny nodules up to 1 mm. in diameter were found at the inoculated sites. These consisted mostly of connective tissue cells, phagocytes, and cartilage. No epithelium had survived. Surface papillomas had

[4] Bullock, F. D., and Rohdenburg, G. L., *J. Med. Res.*, 1915, **33**, 53.

appeared on the skin of the mother where virus had come into contact with it on withdrawing the needle. The experiment was repeated in another animal with identical results: no papillomatous or other progressive growths resulted when 25-day embryo skin, secured after hysterectomy and exposed to the virus *in vitro* was implanted into the muscles and subcutaneous tissue of the mother. In previous tests it had been found that implanted embryo epithelium survives and proliferates for several weeks before disappearing.

The results of several collateral experiments emphasize the significance of these tests. Minced portions of normal skin procured from 8 adult, domestic rabbits were exposed *in vitro* to the virus in the same way as was the embryo skin, and auto-implantations of the minced skin were made as before into the muscles and subcutaneous tissues. In every case papillomatous growths developed which were precisely like those resulting from auto-implantation of the Shope papilloma. On the other hand, attempts to infect Brown-Pearce tumor cells with papilloma virus by the same procedure yielded negative results.[5]

Summary. It is plain that the Shope papilloma virus is remarkably specific in its action, affecting only the epidermis of rabbits and hares and failing to influence embryonic epidermis or other kinds of epithelium, even when this is keratinizing as the result of avitaminosis A, or proliferating in consequence of Scharlach R stimulation.

9009 P

A Virus Causing Oral Papillomatosis in Rabbits.

ROBERT J. PARSONS AND JOHN G. KIDD. (Introduced by Peyton Rous.)

From the Laboratories of the Rockefeller Institute for Medical Research, New York.

Papillomas can often be found in the mouths of domestic rabbits. They are usually situated on the under surface of the tongue, rarely elsewhere on the buccal mucous membrane. We have found them in 67 (17.4%) of 385 gray-brown, Dutch-belted, New Zealand, Havana and chinchilla rabbits obtained from various local sources. None was present in 44 Kansas cottontails.

[5] Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 79.

The growths occur as discrete, sessile or pedunculated, rugose, filiform or fungiform projections. They are usually multiple, often numerous, and their translucent, gray-white color contrasts sharply with the pink mucosa. They may be 5 mm. across and 4 mm. high, though usually less than half this size.

Microscopically the papillomas consist of folded and greatly thickened epithelium supported upon narrow connective tissue cores. Some coarse branching of the cores is present in the larger growths. Invasion of the underlying structures has not been observed. Mitoses are very frequent in the affected epithelium and both cells and nuclei are abnormally large. No stratum granulosum can be discerned and the cells desquamate when but slightly flattened, and while their nuclei are still notably basophilic. There is a noteworthy absence of inflammatory changes in the supporting connective tissue. No definite inclusion bodies have been found on search. In most animals the growths have retrogressed after some weeks but in some they have persisted unchanged, in one instance for 145 days—the maximum period of observation.

The disease can readily be transmitted by means of Tyrode extracts of fresh, glycerinated, or frozen and dried papillomas. Tattoo inoculation of such extracts into the under surface of the tongues of 59 domestic rabbits has yielded growths in 48 individuals, while all of 32 western cottontails have proved susceptible. Preservation of the papilloma tissue in 50% glycerol for 84 days, as also drying it in the frozen state, has entailed no perceptible loss in its potency.

Filtered extracts of fresh or glycerinated papillomas have regularly proved capable of causing the disease in series after passage through Berkefeld V and N candles. For example, glycerinated papillomas which had been obtained from the tongues of 18 domestic rabbits, were trimmed, pooled and ground in a mortar with sterile sand. Tyrode's solution was added to make a 2% suspension. The clear supernatant fluid obtained after light centrifugalization of this suspension was passed through a Berkefeld V candle which was tested concurrently with *B. prodigiosus*. The culturally sterile filtrate was tattooed into the under surface of the tongues of 7 domestic and 3 cottontail rabbits. By the nineteenth day papillomas had appeared in all the cottontails and in 6 of the domestic rabbits. After 31 days the papillomas from 2 of the cottontails and 1 of the domestic rabbits were pooled and treated as above. The bacteriologically sterile fluid procured by passing the Tyrode suspension through a Berkefeld V candle induced growths in all of the 5 domestic rabbits inoculated therewith.

Control inoculations of the buccal mucous membrane with sterile Tyrode's solution or with Shope papilloma virus in domestic rabbits and cottontails have not produced papillomas.

In general, papillomas have appeared from 9 to 38 days after inoculation, the incubation period varying with the concentration of the inoculum. The experimentally induced growths are usually confluent or semi-confluent along the lines of inoculation, as would follow from the method employed. They are otherwise precisely like the naturally occurring growths. If the neighboring mucosa has been accidentally traumatized during inoculation, papillomas may appear here as well.

The agent causing the papillomas shows a remarkable tissue affinity, regularly inducing growths on the under surface of the tongue but rarely where introduced on the dorsum of the organ or on the floor of the mouth, and never, thus far, when inoculated on the mucous membrane of the nose, conjunctiva, genitals, or the skin of the lip and abdomen. Inoculation of the active agent into the oral mucous membrane of dogs, guinea pigs, rats and mice resulted in no lesions.

Animals carrying the papillomas or in which they have recently retrogressed, generally prove insusceptible on inoculation with potent papilloma extracts. Cottontails carrying the Shope papilloma or proving resistant on inoculation with the virus responsible for these growths invariably prove susceptible to oral papillomatosis on experimental inoculation, but some domestic rabbits have proved resistant, the proportion being no greater, however, than in hosts previously unexposed to the Shope virus.

Summary. Oral papillomatosis is common in domestic rabbits bred in the New York area. The disease is due to an hitherto undescribed virus which is evidently pathogenic for the oral mucous membrane only.

9010 P

Distribution of Sulfur in Crystalline Insulin.

BEATRICE KASSELL AND ERWIN BRAND.

From the Department of Chemistry, N. Y. State Psychiatric Institute and Hospital, New York.

The distribution of the sulfur in insulin has long been a subject of interest. Crystalline insulin contains 3.06 to 3.38% of sulfur.[1] Only part of the total sulfur could be accounted for by cystine, the Folin-Marenzi method yielding 9-10% and the Sullivan method 8-9% of cystine.[2] Methionine has been claimed to be absent.[2, 3]

A reliable technique for the estimation of methionine in proteins has been established by Baernstein[4] and further developed to include the iodometric determination[4] of cysteine and homocysteine. The Folin method for the determination of cystine has been adapted to the Pulfrich Photometer,[5] resulting in greater specificity. It has been found that the Sullivan method gives more constant and reliable results if the time interval between additions of color reagent and sulfite is regulated[6] (10 seconds measured with a stopwatch).

It was, therefore, thought desirable to determine the distribution of the sulfur in crystalline insulin with the aid of these improved techniques. A sample of such material (crystalline insulin, Lilly T-800) was kindly put at our disposal by Eli Lilly and Company. The insulin was repeatedly extracted with petroleum ether and dried *in vacuo* at 100°C. Its sulfur content (Pregl) was found to be 3.11 and 3.14%. The ash content was 1.5%. Certain observations[7] may indicate that 1-3% of the total sulfur of this preparation is in the form of sulfate. The presence or absence of such traces of sulfate could not be ascertained directly on account of the large amounts of crystalline insulin necessary.

The experimental data are presented in Table I. The determinations by Baernstein's methods were carried out as described,[4] ex-

1 Scott, D. A., *J. Biol. Chem.*, 1931, **92**, 281.

2 *cf.* Jensen, H., Evans, E. A., Pennington, W. D., and Schock, E. D., *J. Biol. Chem.*, 1936, **114**, 199.

3 Freudenberg, K., Dirscherl, W., and Eyer, H., *Z. physiol. Chem.*, 1930, **187**, 89.

4 Baernstein, H. D., *J. Biol. Chem.*, 1934, **106**, 451; *J. Biol. Chem.*, 1936, **115**, 25, 33.

5 Kassell, B., *J. Biol. Chem.*, 1935, **109**, xlix.

6 Rossouw, S. D., and Wilken-Jorden, T. J., *Onderstepoort J. Vet. Sci.*, 1934, **2**, 361.

7 Kassell, B., and Brand, E., unpublished experiments.

TABLE I.
Cystine and Methionine Analysis of Crystalline Insulin.
(Lilly T-800, Total S—3.13%, Ash—1.5%.)

Exp. No.	Insulin mg.	Hydrolysis‡ Acid used 5 cc.	Time hr.	Cystine Sullivan %	Folin (Photo.) %	Baernstein %	Methionine (vol. iodide) %
1	51.8	6N-HCl	6	10.5	10.8		
2	32.6	6N-HCl	8	10.8	11.0		
3	28.7	6N-HCl	17	11.5	11.1		
4	45.4	57% HI	6			11.2*	0.8†
5	157.6	57% HI	6			11.2*	0.6†
Average				10.9	11.0	11.2	0.7
Calculated as S					2.94		0.15
Percent of Total S					94		5

*Corrected for decomposition (H_2S ≏ 0.8% Cystine).[7]
†Corrected for methyl mercaptan formation[7] ≏ 0.05% Methionine.
‡Bath temperature for HCl—130°C., for HI—150°C.

cept for some minor modifications and corrections.[7] The small amounts of insulin used in the estimations precluded the accurate determination of homocysteine, but the presence of its lactone in the HI digests was established. There were no sulfhydryl compounds in the HCl hydrolysates, and the modified Folin photometric determination[5] indicated the probable absence of disulfides other than cystine.

The average cystine content of the hydrolysates was 11.0%. The Sullivan method, the Folin photometric method, and the Baernstein method gave practically identical results. The figures seem to indicate that hydrolysis for 6-8 hours liberates all of the cystine.

The methionine content of the preparation was 0.7%. This amount is so small that, to exclude the possible presence of methionine-containing impurities in our sample, further experiments on different and specially recrystallized samples of crystalline insulin may be necessary.

In these experiments 99% of the total sulfur of a preparation of crystalline insulin was accounted for, 94% by cystine and 5% by methionine. It is interesting to note that the sulfur distribution of insulin resembles that of wool.[8]

[8] Barritt, J., *Biochem. J.*, 1934, **28**, 1.

Note: Drs. duVigneaud and Miller informed us that their unpublished experiments indicate a cystine content of crystalline insulin higher than heretofore obtained.

9011 P

Hemodynamics Following Experimental Coronary Occlusion in Dogs.*

LOUIS GROSS, MILTON MENDLOWITZ† ‡ AND GERHARD SCHAUER.
From the Laboratories of the Mount Sinai Hospital, New York, N. Y.

After a series of preliminary studies on dogs, a technique was instituted with which it was possible to carry out, within a period of approximately 30 minutes, a variety of experimental procedures which reflect the hemodynamics of the circulation under various conditions. The following factors were studied: cardiac output, total blood volume, venous and arterial pressure, ether and cyanide circulation time, hemoglobin percentage, erythrocyte count, serum proteins, pulse rate and temperature. Table I lists the findings in the following 5 groups of dogs, each group representing 10 animals:

1. Anesthesia controls. In this group, studies were made 15 minutes after the administration of anesthesia and one-half hour later.

2. Thoracotomy controls. In this group, thoracotomy was performed under anesthesia, the pericardium was opened and the left anterior descending coronary branch was dissected but not tied. The hemodynamic studies were made immediately before the thoracotomy and immediately after closing the chest.

3. Sudden left anterior descending coronary branch ligation. In this group, hemodynamic studies were made under anesthesia immediately before the thoracotomy and vessel ligation and immediately after closure of the chest.

4. Double carrick-bend controls. In this group, a special knot (double carrick-bend) was placed around some relatively avascular left ventricular muscle near the left anterior descending coronary branch. The threads from the knot were led out at opposite points in the chest wall. One week later, these muscle fibers were ligated by traction on the threads from the exterior of the chest. Studies were done under anesthesia before and after this control ligation.

5. Double carrick-bend left anterior descending coronary branch ligation. In this group, the knot was placed around the artery. One week later, hemodynamic studies were made under anesthesia

* Aided by grants from the Lucius N. Littauer and Walter W. Naumburg Funds.

† George Blumenthal, Jr., Fellow.

‡ Aided by a grant from the Emanuel Libman Fellowship Fund.

before and after occlusion of the vessel by traction on the threads emerging from the chest.

TABLE I.
Average Immediate Changes in the Circulatory Dynamics Following Various Procedures under Anesthesia.

Procedure:		1. Anesthesia Controls	2. Thoracotomy Controls	3. L.A.D. Coronary Branch Ligations	4. Double Carrick-Bend Controls	5. Double Carrick-Bend L.A.D. Coronary Branch Ligations
Cardiac Output:	Pre.*	100	100	100	100	100
% changes per	Post.*	86	82	52	83	56
sq. meter	Diff.	—14	—18	—48	—17	—44
Total Blood Vol.:	Pre.	100	100	100	100	100
% changes per	Post.	98	92	96	96	89
sq. meter	Diff.	—2	—8	—4	—4	—11
Arterial Blood	Pre.	123	142	146	122	118
Pressure: mm.	Post.	125	121	117	120	120
of mercury	Diff.	+2	—21	—29	—2	+2
Venous Pressure:	Pre.	3.0	2.3	2.7	1.8	1.3
cm. of water	Post.	2.7	1.8	2.0	2.0	1.3
	Diff.	—0.3	—0.5	—0.7	+0.2	0
Ether Circulation	Pre.	4.6	3.4	4.7	4.6	4.3
Time: sec.	Post.	4.1	4.9	5.2	5.2	5.2
	Diff.	—0.5	+1.5	+1.5	+0.6	+0.9
Cyanide Circula-	Pre.	7.6	8.8	9.2	11.1	8.7
tion Time: sec.	Post.	8.0	11.1	14.8	11.6	12.8
	Diff.	+0.4	+2.3	+5.6	+0.5	+4.1
% Hemoglobin	Pre.	77	87	81	82	71
	Post.	81	90	82	83	74
	Diff.	+4	+3	+1	+1	+3
Erythrocyte count	Pre.	6.31	5.95	5.88	6.32	5.53
in millions per	Post.	6.85	6.40	5.82	6.41	6.21
cu.mm.	Diff.	+0.54	+0.45	—0.06	+0.09	+0.68
Total Serum	Pre.	5.65	6.39	5.91	5.07	5.18
Proteins	Post.	5.63	6.51	6.17	4.99	5.25
	Diff.	—0.02	+0.12	+0.26	—0.08	+0.07
Pulse Rate per	Pre.	161	187	176	168	180
min.	Post.	167	159	152	168	155
	Diff.	+6	—28	—24	0	—25
Temperature (F)	Pre.	101.7	100.9	99.8	101.8	99.5
	Post.	101.0	100.7	98.7	101.3	97.7
	Diff.	—0.7	—0.2	—1.1	—0.5	—1.8

*Pre. and Post. refer to pre- and post-operative procedures in groups 2 and 3. In group 1 studies were made 15 and 45 min. respectively after induction of anesthesia. In groups 4 and 5 they were made before and after ligation of the muscle or artery.

As can be seen from the table, a significant immediate change following the respective procedures using the anesthesia control group as a baseline, was a fall in the average cardiac output after ligation of the left anterior descending coronary branch both in the open chest as well as in the closed chest (double carrick-bend). Whereas the other control groups also showed a diminution in the average cardiac output, this diminution was considerably smaller than in animals in which coronary ligation had been performed. The only other significant change was a prolongation of cyanide circulation time in both groups in which coronary branch ligation was done.

Fluctuations in arterial blood pressure were considerable. However, a relatively small fall in the average arterial blood pressure occurred only in those groups in which the chest had been opened. Since this also took place in the thoracotomy control group, the fall in blood pressure could not be attributed to the coronary branch ligation. No appreciable differences were found in venous blood pressure. The total blood volume, ether circulation time, hemoglobin percentage, erythrocyte count and total serum protein showed insignificant changes. There was a moderate slowing in pulse rate in the open chest operations as well as in the double carrick-bend ligations. This, however, was insufficient to account for the change in cardiac output. There was apparently some relation between the drop in temperature and the decreased cardiac output.

Conclusion. Following left anterior descending coronary branch ligation in the open or closed chest there is an immediate appreciable fall in average cardiac output and also a delay in cyanide circulation time. Control experiments indicate that these changes are directly attributable to the ligation of the coronary branch. Following these procedures there are no other appreciable changes in the hemodynamics studied.

9012 C

Preparation of Gliadin and Zein.*

LAURENCE S. NOLAN AND HUBERT BRADFORD VICKERY.

From the Biochemical Laboratory, Connecticut Agricultural Experiment Station, New Haven.

In the course of many years of experience in the preparation of gliadin and zein of a quality suitable for nutrition investigations, convenient and relatively simple methods to obtain these proteins have been devised. So many requests for these methods have been received that publication seems desirable.

Gliadin from Wheat Gluten Flour. Approximately 1 kilo of wheat gluten flour is sifted with vigorous stirring into 7 liters of 70% alcohol at 55-60°C. The mixture is allowed to digest for about 30 minutes and is then poured through a fairly fine cotton gauze (cheesecloth) supported on a flat wire-mesh rack of sufficient area so that not too thick a layer of hydrated gluten is formed and the alcohol runs through freely. After being allowed to drain, the gluten is extracted twice more with 5 liter lots of hot 70% alcohol. The residue in convenient portions is then folded tightly into square cakes in light canvas or drilling press cloth. These are pressed, two or more at a time, between steel plates in the hydraulic press.

The extracts are combined and filtered through a dense pad of paper pulp (4-5 cm. thick) on a large Buchner funnel. The pad is made with an aqueous suspension of pulp and is finally washed with diluted alcohol. The filtrate should be perfectly clear. This filtrate is concentrated *in vacuo* until frothing can no longer be controlled; the addition of an occasional few drops of octyl alcohol is of material assistance toward the end of this operation.

The clear concentrated alcoholic solution is placed in lots of about 1 liter in a large enamelware tub, and water is violently squirted into the tub in such a manner as to cause the greatest possible agitation. Under these conditions, the gliadin separates from solution in the form of a highly aerated froth. This is skimmed off, and, when all has been collected, is thoroughly beaten or whipped with a wire cream beater until homogeneous. The froth is then placed in a thin layer (3-5 cm.) on pans and is rapidly dried in a current of warm air (70-80°C.). After the froth has become somewhat dry on top, any water that has accumulated beneath it is poured off.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

The drying must be rapid in order to avoid a collapse of the froth; when correctly dried the product is a crisp, white, much expanded mass. It is crushed by hand and passed through a mill to reduce it to a light somewhat scaly powder.

Gliadin has been prepared in this laboratory by various modifications of this procedure for the past 14 years. The product contains about 15.5% of nitrogen calculated ash- and moisture-free, and is entirely suitable for nutrition studies in which such phenomena as the stunting of growth due to lysine deficiency are to be demonstrated.

Zein from Corn Gluten. Five kilos of dry and finely ground corn gluten (preferably from white corn) are stirred into 20 liters of hot 80% alcohol (50-60°C.) and the mixture is kept hot for about 30 minutes. Filter paper clippings are then added with stirring until a mass is produced that is stiff and dry enough to be molded into cakes which can be folded in canvas press cloth and pressed. The extract obtained from the press is highly colored and quite viscous. It is filtered through a thick pad of paper pulp made on a Buchner funnel as already described. Filtration is slow, but a perfectly clear filtrate is obtained.

The filtrate is treated in convenient portions with an equal volume of ether, and the precipitated protein is thoroughly stirred with the fluid which is finally poured off. This step removes most of the fat and pigment and is less troublesome than the extraction with ethylene dichloride advocated by Mason and Palmer.[1] Reprecipitation of an alcoholic solution of the protein with ether may be carried out if a product of high purity is sought.

The precipitated protein is dissolved in a small amount of warm 80% alcohol and should form a perfectly clear, although very thick and viscous solution. This is transferred to a separatory funnel with the aid of a little diluted alcohol, and the funnel is set up over the following device. A large sheet of cotton gauze (cheesecloth) is spread on a wire-mesh rack in a sink and on it is placed a shallow round pan (milk-pan). Water from a hose which has been fitted with a glass nozzle constricted so as to deliver a rapid flat stream about 2 cm. wide is directed into this pan. A *very thin continuous* stream of the zein solution is then allowed to fall from the separatory funnel upon this stream of water. The zein is partially precipitated at contact and the process is completed in the violently agitated water in the pan. Most of the protein is washed out of the pan but is retained by the gauze. At the end of the operation it

[1] Mason, I. D., and Palmer, L. S., *J. Biol. Chem.*, 1934, **107**, 131.

is collected in the gauze, thoroughly washed with water and is finally allowed to remain in water over night. It is then filtered on a Buchner funnel, spread in a thin layer on pans and allowed to dry at room temperature. Before it becomes thoroughly dry, there is a point at which it can be easily rubbed through a fine sieve whereby a uniform product is obtained. After being thoroughly dried in the air, it is ground to powder in a mill. Such preparations contain approximately 16.2% of nitrogen, ash- and moisture-free, and have been used with success in experiments to demonstrate the peculiar nutritive deficiencies of this protein.

Summary. Methods are described for the preparation of gliadin from wheat and of zein from corn (maize). The products are of a grade suitable for many types of nutrition investigations.

9013 P

Blood Chemistry of the Chick Embryo During Ontogenesis.

CARLA M. ZORN AND ALBERT J. DALTON. (Introduced by V. C. Myers.)

From the Departments of Biochemistry and Histology, School of Medicine, Western Reserve University.

Within the last few years increasing attention has been given to studies concerning the changes in the concentration of a number of blood constituents during prenatal development. With the aid of such data it may be possible to add further information concerning the utilization of the various foodstuffs during the embryonic period. Further, it may be possible, by correlating the extent of functional development of the various organs of the embryo at a given age with such chemical blood data, to throw further light upon the influence of a given organ upon intermediary metabolism.

The chick embryo was chosen as the experimental animal. The eggs were incubated under standard conditions and at the desired age the egg was carefully opened and sufficient blood for analysis was withdrawn from the vitelline artery or vein into a 1 cc. tuberculin syringe. Since satisfactory blood samples could not be obtained from embryos of less than 9 days incubation no analyses were attempted until that time. From this period on, however, the analyses were carried on through the entire incubation period and for several days after hatching.

TABLE I.

The Changes in the Blood Chemistry of the Chick Embryo During Ontogenesis. Average values. No. of embryos and determinations in parentheses.

Days from Beginning of Incubation	9	10	11	12	13	14	15	16	17	18	19	20	21*	22*	23*	24*	29*
Blood Sugar mg./100 cc.	143	126	143	143	131	137	133	158	157	161	158	170	182	229	206	246	
	(4)	(4)	(4)	(5)	(7)	(6)	(4)	(4)	(6)	(7)	(3)	(2)	(3)	(2)	(1)	(2)	
Uric Acid mg./100 cc.	1.34	1.59	1.72	1.83	1.62	1.87	2.39	2.35	2.20	2.13	2.66	2.71	2.94	5.01	4.34	7.22	4.95
	(2)	(2)	(2)	(2)	(5)	(2)	(3)	(2)	(4)	(3)	(3)	(5)	(6)	(4)	(1)	(1)	(2)
Cholesterol mg./100 cc.	98	156	164	175	173	192	296	274	360	364	313	207	172	241		249	
	(2)	(2)	(2)	(1)	(4)	(3)	(4)	(2)	(3)	(3)	(3)	(2)	(4)	(5)		(4)	
Hemoglobin gm./100 cc.	6.6	6.3	6.5	9.3	10.1	12.7	14.1	14.0	13.1	13.2	11.2	11.4	12.2	10.9	10.6	9.9	9.8
	(4)	(4)	(2)	(4)	(6)	(3)	(2)	(3)	(6)	(3)	(9)	(6)	(7)	(2)	(1)	(7)	(2)
Red Blood Cells million/cmm.	0.95	0.73	1.06	1.11	1.33	1.66	1.79	1.88	2.20	2.15	1.89	1.96	2.08	2.61	2.00	2.35	1.80
	(2)	(2)	(2)	(2)	(4)	(2)	(3)	(2)	(1)	(2)	(3)	(4)	(2)	(2)	(2)	(3)	(1)

*Hatched chicks were used on these days.

Table I presents the results of the average daily changes occurring in the several constituents of the blood during the course of ontogenetic development. The numbers in parentheses indicate the number of separate analyses from different embryos used to compute the average. From these results it is observed that the embryonic blood sugar is increased beginning with the 16th day of incubation. The increase in the uric acid concentration is observed much earlier in the developmental period. Similar increases are observed in the concentration of cholesterol and hemoglobin, and in red blood cell counts, with the exception of a definitely lowered level during the hatching period.

Further studies are in progress to determine more exactly the extent and significance of these changes.

9014 P

Availability of Iron in Wheat.

ALFRED H. FREE AND FRANKLIN C. BING.

From the Department of Biochemistry, School of Medicine, Western Reserve University.

Elvehjem, Hart, and Sherman[1] have reported that the inorganic iron content of wheat is approximately 47% of the total iron content. Their biological studies indicated that this figure also represents the available iron content. On the contrary Rose, Vahlteich, and MacLeod[2] observed that wheat is an excellent source of iron for hemoglobin formation.

In order to determine whether there is any great variation in the form of iron in different varieties of wheat, 11 samples representing both hard spring wheat and soft winter wheat were analyzed for total and inorganic iron. A modification of the technic of Elvehjem, Hart, and Sherman,[1] which employs a longer extraction period, was used to determine the inorganic iron, whereas the total iron was determined on ashed samples by the thioglycolic acid method.[3] The total iron of the 11 samples ranged from 2.90 mg. to 4.87 mg. of iron per 100 gm. of wheat. The inorganic iron showed

[1] Elvehjem, C. A., Hart, E. B., and Sherman, W. C., *J. Biol. Chem.*, 1933, **103**, 61.

[2] Rose, M. S., Vahlteich, E. M., and MacLeod, G., *J. Biol. Chem.*, 1934, **104**, 217.

[3] Hanzal, R. F., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 846.

about the same amount of variation, the range being from 2.46 mg. to 4.04 mg. of iron per 100 gm. of wheat. The percentage of inorganic iron with respect to the total iron varied from 73% to 88%, the average being 81%. This figure agrees fairly well with the value suggested by Shackleton and McCance.[4]

Two of the samples of wheat were also used for a biological assay. Albino rats were made anemic by employing an exclusive milk diet. As soon as values below 4.0 gm. of hemoglobin per 100 cc. of blood were attained, the experimental diets were started. Three groups of experimental animals received 0.25 mg. of iron per day. One group of 5 animals received 0.25 mg. of iron per day furnished by $FeCl_3$; a second group of 6 animals received Trumbull wheat (soft winter wheat) in quantities to supply the same amount of iron per day; and the third group of 6 animals received Nabob wheat (soft winter wheat) in quantities to supply 0.25 mg. of iron per day. All of the animals were given adequate supplements of copper and manganese along with as much milk as they would drink. The hemoglobin levels were determined at weekly intervals for a 4-week period. The actual hemoglobin increase is calculated by subtracting the initial hemoglobin concentration from the final hemoglobin concentration. In each group of experimental animals the actual increase in hemoglobin concentration for the 4-week period was between 9.1 and 10.1 gm. of hemoglobin per 100 cc. of blood.

The actual increases in hemoglobin concentration for each group of experimental animals are not significantly different. The results show that the iron of wheat is well utilized for hemoglobin formation. If it can be assumed that the iron of $FeCl_3$ is all available, then it appears from these animal experiments that the iron of wheat is practically all available.

[4] Shackleton, L., and McCance, R. A., *Biochem. J.*, 1936, **30**, 582.

9015 P

Creatine, Potassium and Phosphorus Content of Cardiac and Voluntary Muscle.*

GEORGE H. MANGUN AND VICTOR C. MYERS.

From the Department of Biochemistry, School of Medicine, Western Reserve University.

The study of the creatine content of human voluntary and left and right ventricular cardiac muscle, carried out by Seecof, Linegar and Myers,[1] has been extended to include determinations of potassium and phosphorus. It is evident from the studies of Fiske and Subbarow[2] and others that in resting muscle creatine exists largely as phosphocreatine. It is likewise evident that this compound must be bound to some base, and this would appear to be largely, if not wholly, potassium.

At about the time we began our original study on the creatine of heart muscle, Calhoun, Cullen, Clarke and Harrison[3] pointed out that a diminished potassium content of heart muscle was invariably present in failing ventricles and suggested that this change may have been a contributing cause of such failure. Linegar and Myers[4] noted that the creatine concentration also drops in heart failure and would appear to parallel very closely the observations of the Vanderbilt investigators on potassium. It was then suggested that this drop in potassium and creatine may represent 2 phases of the same process.

Studies have now been carried out on 72 human autopsy cases in which potassium and phosphorus have been estimated in addition to the creatine. Since the cases were complicated by wide variations in diagnosis, it seemed advantageous to group them according to heart weights, excluding cases with nitrogen retention, which may cause elevated creatine values. The data studied were compiled from 44 hearts ranging in weight from 200 to 825 gm., comparable changes being found in both left and right ventricular muscle.

It was noted that as the heart weight progressively increases there was a diminution in the concentration of the 3 constituents, creatine,

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Seecof, D. P., Linegar, C. R., and Myers, V. C., *Arch. Int. Med.*, 1934, **53**, 574.

2 Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1929, **81**, 629.

3 Calhoun, J. A., Cullen, G. E., Clarke, G., and Harrison, T. R., *J. Clin. Invest.*, 1930, **9**, 393.

4 Linegar, C. R., and Myers, V. C., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1016.

potassium and phosphorus, the ratio of the average decrease being 3 : 2 : 1 respectively. This same sequence was found in the majority of cases, although in some, all 3 constituents decreased in the same proportion, due possibly to dilution of the muscle elements as a result of pathological change. This is not the rule, however, and if it may be assumed that in resting muscle creatine exists as the dipotassium salt of phosphocreatine, this would help to explain the ratios found in the loss in concentration since they correspond roughly with the intramolecular ratios found in this hypothetical compound. The loss of potassium and phosphorus is somewhat greater than that required by this hypothesis, but it is to be expected since the amount of creatine is quite insufficient to combine with all the potassium and phosphorus present in heart muscle.

9016 C

Effect of Oxygen on *Bacterium necrophorum* in the Isolated Colon Segment of a Dog.

FRANCIS O. FRY AND G. M. DACK.

From the Department of Physiol. and Bacteriology, University of Chicago.

Oxygen has been employed in the experimental treatment of ulcerative colitis.[1, 2] In view of the almost constant association of *Bact. necrophorum* with this disease,[3, 4, 5] and the sensitivity of this organism to oxygen,[6] it seemed advisable to study the effect of oxygen on cultures introduced into the colon.

For these experiments a dog was used which had been operated upon and an isolated colonic segment prepared. This loop included about 7 8 cm. of terminal ileum, the open end of which was brought out through a stab wound in the abdomen, providing an entrance to the colonic segment. The loop consisted of caecum, the ascending and one half of the transverse colon. The transverse colon was divided, the proximal opening closed with a purse string suture, and the distal open end anastomosed end to side to the small bowel to reestablish the flow of faecal current. The only exit of material

1 Polsen, Joseph, *Arch. Int. Med.*, 1931, **48**, 736.

2 Golob, Meyer, *J. Am. Med. Assn.*, 1936, **106**, 1735.

3 Dack, G. M., Reins, T. E., and Dragstedt, L. R., *Arch. Surg.*, 1935, **31**, 225.

4 Dack, G. M., Dragstedt, L. R., and Reins, T. E., *J. A. M. A.*, 1936, **106**, 7.

5 Dack, G. M., Dragstedt, L. R., and Reins, T. E. In press.

6 Beveridge, *J. Path.*, 1934, **38**, 467.

from this loop of colon was through the stump of ileum which went to the outside. We have found this type of bowel-segment to accommodate considerable amounts of fluid without appreciable leakage. Material was introduced and withdrawn from the bowel by introducing a sterile, soft rubber urethral catheter into the bowel.

The following general technic was used for the whole experiment. A strain (101) of *Bact. necrophorum* was used which had been isolated at proctoscopic examination from the colon of a patient with severe ulcerative colitis. The cultures used in the experiments were grown in deep tubes of Rosenow's glucose-brain medium for 24 hours at 37°C. The supernatant fluid from these cultures was centrifugalized to concentrate the organisms. Part of the clear, centrifugalized, supernatant was discarded and the sediment suspended in the remaining portion, making a total volume of 40-50 cc.

The culture thus prepared was introduced into the colon. At intervals of 30 minutes, 1, 2, 4, 8, 12, etc., hours, specimens were removed. Decimal dilutions of from 1-10 to 1-1 billion were made in 9 cc. blanks of cystine (0.05%), dextrose (1%), veal-infusion broth. This diluting fluid was used because it does not appear to impair the viability of *Bact. necrophorum*. One cc. of the various dilutions was then pipetted into tubes of Rosenow's glucose-brain medium. The tubes were incubated at 37° C. and blood agar plates streaked from those showing turbidity and gas in 24 hours. The inoculated plates were incubated in anaerobic jars as previously described,[4] and examined after 48 hours' incubation at 37°C. *Bact. necrophorum,* if present in the dilutions cultured, was recognized by the green zone of hemolysis surrounding the colonies. This green zone developed several minutes after removal of the plate from the anaerobic jar. Confirmation of these colonies as *Bact. necrophorum* was established by microscopic examination and finding long filamentous, pleomorphic, granular forms. Characteristic colonies were picked and put back in Rosenow's medium to find out whether or not the growth and morphology remained typical. All cultures were tested on aerobic blood-agar slants to make sure that the recovered strains were true anaerobes.

By the use of this technic the relative concentration of *Bact. necrophorum* could be determined and any marked variation in their number thus easily observed. In all cases, before the suspension was introduced into the colon, the number of viable organisms was determined by the method previously described.

A determination was made of the rate of disappearance of *Bact. necrophorum* from the normal colon. This was done by introducing

cultures into the colon and withdrawing samples at various intervals and subjecting them to the procedure described. Thus, it was learned that *Bact. necrophorum* survived in a viable form for a length of time exceeding 12 hours, but less than 24 hours. (Table I.)

TABLE I.

Rate of Disappearance of *Bact. necrophorum* Experimentally Introduced into the Isolated Colon of Dog.

No. of Viable Organisms in Colon Specimen (Millions per cc.).

Exp.	No. Viable Organisms per cc. of inoculum	½	1	2	4	8	12	16	24 hr.
1	1	1	1	1	.1	.1	*	*	0
2	1	1	1	1	1	.1	.1	.01	0
3	1	1	.1	.1	.1	.1	.01	.01	0
4	10	.1	.1	.1	.1	.01	.01	*	0
5	1	1	1	1	.1	.1	.1	*	0

*Not determined.

The influence of oxygen upon *Bact. necrophorum* in the colon was studied; 50 cc. bacterial suspension were put into the loop and 5 minutes later oxygen was bubbled into it. The oxygen was obtained from a cylinder, the amount regulated by a valve, and was led into the colon through a sterile catheter after first being bubbled through water, in order to measure the volume of gas admitted. It was established that 18 bubbles expelled 1 cc. of water from a graduated cylinder. About 120 bubbles were injected per minute, over a period of 90 minutes, making a total of about 600 cc., some of which escaped through the fistula. Samples of loop-contents were then withdrawn and tested.

Bact. necrophorum were no longer demonstrable in the colonic segment after 90 minutes' treatment with oxygen.

To prove that the disappearance of the organisms was due to the oxygen and not to any mechanical action, such as distension of the colon and increased secretion and peristalsis, nitrogen was substituted for oxygen under identical conditions. The inert gas, nitrogen, did not affect the viability of *Bact. necrophorum*.

Cultures of *Bact. necrophorum*, in Rosenow's medium, were tested *in vitro*. Oxygen was bubbled through the medium at the rate of 120 bubbles per minute, and then samples were tested at intervals as before. It was found that oxygen for 90 minutes caused a great diminution in the number of these organisms, although 6 hours of this treatment was necessary to kill all of them.

Summary. *Bacterium necrophorum* introduced in large numbers into the isolated colon of a dog were recovered in appreciable num-

bers after a period of 12 hours. When oxygen was admitted into the bowel containing the culture for a period of 90 minutes no *Bact. necrophorum* organisms were recovered at the end of that time. Nitrogen similarly injected into the bowel was without effect.

9017 C

Absence of Vitamin E in the Royal Jelly of Bees.*

K. E. MASON AND R. M. MELAMPY.

From the Department of Anatomy, Vanderbilt University School of Medicine, Nashville, Tenn., and the Bureau of Entomology and Plant Quarantine, United States Department of Agriculture.

In 1925, one of the writers (K.E.M.) working under the direction of the late Drs. T. B. Osborne and L. B. Mendel at the Connecticut Agricultural Experiment Station, attempted to assay the vitamin E-content of the royal jelly of the honey bee, which is the substance necessary for transformation of worker larvae into the queen or sexually productive form. Due to the relatively small amount of material procurable at the time, and to the negative data obtained, the results of these studies were not published. Since that time, Hill and Burdett[1] in England, claim to have demonstrated the presence of appreciable amounts of vitamin E in this interesting substance. However, there are many obvious objections to the method of assay used, and to the interpretations of results obtained by the latter investigators. They assume in the first place that, in normal stock females placed upon an E-deficient diet at the time of parturition, the process of suckling the litter would completely remove the stores of vitamin E in the maternal tissues. The fallacy of such an assumption is clear to all those who have had experience in the experimental production of vitamin E-deficiency. Furthermore, they state that out of 3 rats receiving a daily supplement of 50 mg. of royal jelly over a period of 37 days, 2 females were able to deliver litters of fully developed young. Out of 4 other rats, 3 of which received 2 gm. supplements of honey and pollen and 1 of which received 2 gm. of worker larvae brood comb, daily, only 1

* This investigation was aided by a grant to Vanderbilt University from the Division of Medical Sciences of the Rockefeller Foundation. The royal jelly was furnished by the Southern States Bee Culture Field Laboratory, Baton Rouge, Louisiana.

[1] Hill, L., and Burdett, E. F., *Nature*, 1932, **130**, 540.

rat was able to come to term with delivery of 1 dead fetus. The 3 control rats, fed the E-deficient diet only, failed to conceive. They conclude from their experiments that daily additions of approximately 50 mg. of royal jelly, during the period of one month, are capable of preventing sterility in E-deficient rats. It had previously been shown by Taylor and Nelson[2] that honey is devoid of vitamin E.

In view of the unsatisfactory nature of the experiments of Hill and Burdett, and in view of the frequent reference in the literature to royal jelly as a source of vitamin E, it seemed advisable to reinvestigate this whole question more extensively. To date we have tested varying amounts of royal jelly, using 5 female rats in which from 1 to 2 proven resorptions had occurred. That the sterility in these animals was typical of E-deficiency is adequately demonstrated by (1) the appearance of typical E-deficiency paralysis, as first described by Evans and Burr,[3] at the end of the suckling period in the young delivered by 3 of these rats at the pregnancy period immediately preceding the one in which first resorption occurred, and (2) by the repeated repair of sterility in a large number of rats from the same experimental group after administration of various substances which were being simultaneously tested for their vitamin E-content.

The royal jelly was stored below freezing temperature. The daily supplements were accurately weighed, mixed with yeast to form a pellet and fed to the experimental animal. The animals were closely observed until they had completely consumed the entire pellet. The reproductive history of all the rats was followed by daily vaginal smears from the beginning of sexual maturity to the termination of the experiment. The results obtained from 5 tests with royal jelly, together with that from a few typical tests with other substances, are presented in Table I.

From these data it is quite obvious that as much as one gm. of royal jelly fed daily throughout the period of gestation does not convey sufficient vitamin E to permit the completion of gestation in E-deficient female rats. Possible criticism that administration of the substance as late as the 3rd or 4th day of gestation (rats 597 and 590) might not permit sufficiently early storage of vitamin E to protect against the early resorptive changes of E-deficiency is negated by the fact that several rats (606 and 597) received appreciable amounts of royal jelly prior to beginning of the pregnancy

[2] Taylor, M. W., and Nelson, V. E., PROC. SOC. EXP. BIOL. AND MED., 1929, **26**, 521.

[3] Evans, H. M., and Burr, G. O., *J. Biol. Chem.*, 1928, **76**, 273.

TABLE I.

Rat	No. of successive resorptions prior to the "test" pregnancy	Type of supplement tested	Daily dose Mg.	Total dose Gm.	Days of gestation period when daily supplement was given	Results and general remarks.
607	1*	Royal jelly	50	1.1	1-21	Complete resorption.
597	1	" "	100	2.1	4-21	Delivered 1 dead and 2 living fetuses on 21st day.†
590	1	" "	300	3.6	3-14	Killed on 17th day pregnancy. Six fetal sites present, all in advanced stages of resorption.
549	1	" "	400	4.0	1-9	Killed on 13th day pregnancy. Five fetal sites, all in early stages of resorption.
606	2	" "	1000	19.0	1-20	Killed on 20th day pregnancy. Five fetal sites, all very advanced stages of resorption.‡
592	2	Ether extract wheat germ oil (6 mo. old)	100	2.0	1-20	Killed on 20th day pregnancy. Five normal fetal sites containing normal, living fetuses.
658	2	Cold-pressed wheat germ oil	50	1.05	1-21	Delivered 6 living young on 22nd day.
654	1	Dried spinach	500	10.0	2-21	Delivered 9 living young on 21st day.
668	1	Cold-pressed wheat germ oil	Single intraperitoneal injection of 500 mg. on the 5th day of pregnancy.			Delivered 5 living young on 21st day.

*In the pregnancy prior to the "test" pregnancy, laparotomy was performed on the 22nd day of gestation. 8 advanced resorptions were present and a single living fetus found was removed at the time of operation.

†Examination of the vaginal smear indicated that other fetuses were resorbed during this pregnancy. The next gestation in this rat was not established until 67 days later. Between the 9th and 20th, and the 29th and 36th days of this 67-day period, the rat received a total of 2.6 gm. of royal jelly, administered after several positive matings which later proved, from the study of the vaginal smears, to have been infertile. If royal jelly contains a significant amount of vitamin E there should have been sufficient storage from the jelly supplied during and subsequent to the first "test" pregnancy to prevent resorption during the following pregnancy. However, this rat when autopsied on the 10th day of pregnancy presented 6 placental sites in early stages of resorption. The latter was confirmed by histologic examination.

‡ This rat also received a total of one gm. of royal jelly, following infertile copulation, 3 weeks previous to the experimental test.

period. The record of rat 597 might lead one to conclude that royal jelly contains significant amounts of vitamin E were it not for the convincing results obtained from rats on higher doses of the jelly, and for the fact that, very occasionally, one encounters such instances of an apparent resorption in an E-deficient rat followed by the delivery of a litter in a succeeding pregnancy. It should also be mentioned that this rat was the first, out of a group of 24 rats, to show a resorption, and that one may occasionally mistake a resorption for a pregnancy terminated by delivery of young which are eaten by the mother soon after birth.

Certain of the rats were autopsied during pregnancy in order to conserve upon the supply of royal jelly and in order to assure ourselves, by histologic study of the fetal sites, that we were dealing with typical vitamin E-deficiency. Both gross and microscopic study of the placental sites have adequately confirmed this assumption. It should also be mentioned that 12 littermate sisters of the test rats used, with the same dietary and reproductive records, were permitted to continue pregnancies without the addition of any "test" substances. In all cases these pregnancies resulted in resorptions.

Certain suggestions and claims relating to oestrogenic and gonadotropic actions of vitamin E, and to a specific rôle of vitamin E in the endocrine activity of the sex glands and pituitary,[4, 5] have not been substantiated by more careful experimental studies.[6–9] The majority of experimental data available indicate that vitamin E is specifically needed for nuclear activity and function of cells in general, its lack becoming first manifest in those tissues such as the germinal epithelium of the testis and the developing fetus, where cellular proliferation and differentiation are especially rapid.[6, 7, 10, 11]

One might expect vitamin E to play an important rôle in the rapid cellular proliferation involved rather than in the bestowing of fertility upon what would otherwise be a non-functional form, as newly hatched larvae weigh about 0.1 mg. while 7-day larvae attain a weight of nearly 300 mg. The results of the present study

4 Verzár, F., and coworkers, *Proc. Staff Meet. Mayo Clinic*, 1929, **4**, 351; *Arch. f. d. ges. Physiol.*, 1931, **227**, 499, 511; *Biochem. Z.*, 1931, **240**, 19.

5 Szarka, A., *Arch. f. d. ges. Physiol.*, 1929, **223**, 657.

6 Juhász-Schäffer, A., *Klin. Wchnschr.*, 1931, **10**, 1364; *Ergeb. d. inn. Med.*, 1933, **45**, 129.

7 Mason, K. E., *Am. J. Anat.*, 1933, **52**, 153.

8 Olcott, H. S., and Mattill, H. A., *J. Biol. Chem.*, 1934, **104**, 423.

9 Saphir, W., *Endocrinology*, 1936, **20**, 107.

10 Adamstone, F. B., and Card, L. E., *J. Morphol.*, 1934, **56**, 325, 339.

11 Adamstone, F. B., *Science*, 1934, **80**, 450.

indicate that vitamin E is not concerned in either of the above phenomena and that as much as one gm. of royal jelly of the honey bee is insufficient to cure the sterility of E-deficiency in the female rat.

9018 C

Antigenic Differences in Strains of Human Influenza Virus.

T. P. MAGILL AND THOMAS FRANCIS, JR.

From the Laboratories of the International Health Division, Rockefeller Foundation, New York.

On the basis of earlier studies with mouse and ferret passage virus,[1] it was concluded that the Puerto Rico (PR8) and the Philadelphia (Phila) strains of human influenza virus were immunologically identical, while the swine influenza virus was serologically distinct. The 2 strains of human virus were also indistinguishable from the WS strain of the English workers.[1, 2] After repeated inoculation of ferrets with human influenza virus, however, it was noted that the serum of an animal so treated developed the capacity of neutralizing the swine virus as well.[3] Moreover, the serum of rabbits (a non-susceptible animal species) vaccinated with ferret-passage human influenza virus developed antibodies against both the human and swine viruses, whereas rabbits vaccinated with swine influenza virus produced antibodies which neutralized only the swine virus.[3] Identical results were obtained with horse sera prepared by Laidlaw, Smith, Andrewes and Dunkin.[3, 4] It was suggested, therefore, that the human and swine viruses, while immunologically distinct contained common antigens and that the swine antigenic components were present in the human virus as secondary antigens.[3, 5]

Since it seemed likely that the antibody response of an insusceptible animal might reflect the secondary antigens of the virus more completely than that of the susceptible animal in which antibodies to the primary antigen rather than to the secondary antigen

1 Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1172.

2 Andrewes, C. H., Laidlaw, P. P., and Smith, W., *Brit. J. Exp. Path.*, 1935, **16**, 566.

3 Francis, T., Jr., and Shope, R. E., *J. Exp. Med.*, 1936, **63**, 645.

4 Laidlaw, P. P., Smith, W., Andrewes, C. H., and Dunkin, G. W., *Brit. J. Exp. Path.*, 1935, **16**, 275.

5 Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1936, **63**, 655.

would probably constitute the initial response, further studies were carried out in rabbits using the tissue culture virus as the source of the various strains. Rabbits were inoculated intraperitoneally with 2 cc. of culture fluid containing the PR8, Phila, and the Swine-2 strains, respectively. Blood was taken from the marginal ear vein 9 to 15 days later. Neutralization tests were then performed with the serum and tissue culture virus, employing mice as the test animal. The 3 sera were tested simultaneously against the homologous and 2 heterologous virus strains.

TABLE I.

Neutralizing Capacity of Serum against Homologous and Heterologous Strains of Influenza Virus.

Strain of Culture Virus	Serum of Rabbit Vaccinated with			
	PR8	Phila	Swine	Normal
PR8	0	++	+	d4 ++++
	0	++	++	d4 ++++
	+	+	+	d4 ++++
	0	++	+	d5 ++++
	0	++	+	d5 ++++
Phila	d4 ++++	0	d5 ++++	d3 ++++
	d4 ++++	0	d6 ++++	d3 ++++
	d6 ++++	0	d6 ++++	d4 ++++
	d6 ++++	0	d7 ++++	d4 ++++
	d7 ++++	0	d7 ++++	d8 ++++
Swine	d4 ++++	d5 ++++	0	d5 ++++
	d4 ++++	d6 ++++	0	d6 ++++
	d5 ++++	d7 ++++	0	d6 ++++
	d5 ++++	+++	0	d7 ++++
	d8 ++++	++++	0	d7 ++++

Survivors were sacrificed on 8th day after infection.
0 = No pulmonary lesions.
+ to ++++ = degree of pulmonary involvement.
d4 = Died on 4th day.

It was found that a comparatively high degree of specificity was exhibited by each serum. Table I represents the results of such a test. In each instance a serum completely neutralizes the homologous virus strain; the Phila serum in addition partially neutralizes the PR8 strain but not the swine virus; the anti-swine serum partially neutralizes the PR8 strain of human influenza virus but not the Phila strain. These results were not due to differences in the virulence of the respective strains for mice, as shown by the fact that, measured by lung lesions in inoculated mice, the titer of all 3 strains at the time of the test was 1:1,000.

In Table II the results of another test with the serum of different rabbits bled 9 days after a single intraperitoneal injection of 2 cc. of tissue culture virus are shown. At this time the end-point of

TABLE II.
Titration of Serum against Homologous and Heterologous Strains of Influenza Virus.

Serum of Rabbit						
Vaccinated with virus strain	Tested against virus strain	Dilution of Serum				
		Undil.	1:2	1:5	1:10	1:20
PR8	PR8—160th transfer	S	S	S	S	S
		S	S	S	S	S
		S	S	S	S	S
		S	S	S	S	S
	Phila—160th transfer	d6	d5	d5	d4	
		d7	d6	d5	d4	
		d7	d6	d5	d4	
		d7	d6	d6	d4	
	Swine—149th transfer	d4	d4	d4	d4	
		d4	d4	d4	d5	
		d4	d4	d4	d5	
		d5	d5	d4	d5	
Phila	PR8	d7	d6	d5	d4	
		d8	d6	d5	d6	
		d8	d6	d7	d6	
		S	d8	d7	d7	
	Phila	S	S	S	d8	d3
		S	S	S	d8	d7
		S	S	S	d9	d8
		S	S	S	S	d8
	Swine	d5	d5	d4	d5	
		d5	d5	d4	d5	
		d6	d5	d5	d6	
		d6	d5	d5	d6	
Swine	PR8	S	S	d5	d4	
		S	S	d5	d4	
		S	S	d7	d4	
		S	S	S	d5	
	Phila	d5	d4	d4	d4	
		d6	d4	d4	d4	
		d6	d6	d4	d4	
		d7	d6	d4	d5	
	Swine	S	S	d7	d6	d3
		S	S	d8	d7	d4
		S	S	d8	d7	d4
		S	S	d8	d8	d5

S = Survived (Experiment terminated on 10th day).
d4 = Died on 4th day.

the virus titration in each case was 1:10,000. Titrations of the respective sera were made against the homologous and heterologous strains.

Using survival and death of the mice over a period of 10 days as the criterion of protection, it is seen that the PR8 and Phila sera protected only against their homologous strain, while the anti-swine serum protected equally against the swine and the PR8 strains.

These results, which closely parallel those of Alexander in studies

on the neurotropic virus of horsesickness,[6] indicate a definite serological difference in the PR8 and Phila strains of human influenza virus and are in direct opposition to those previously reported when it was concluded that the 2 strains were serologically identical. The present results apply, however, only under the conditions outlined. When serum is obtained from the same rabbit after a longer interval, with or without an additional injection of virus in the meantime, it is found that cross-protection by the sera against PR8 and Phila strains is clearly demonstrable. The anti-swine virus serum may then also exert a protective action against both human strains although in most instances this effect is most pronounced against PR8. In contrast, however, under the present conditions no instance has been encountered even with late bleedings in which serum derived against human strains of virus has neutralized the swine virus. This would suggest, contrary to previously expressed opinion[5] that the swine virus comprises a more complex antigen than the human strains and that the common antigen which elicits the cross-neutralizing antibodies is more effectively present in the swine virus.

Several possibilities must be considered in interpreting the discrepancies between the present results and those previously reported. The strains of virus have been grown in tissue culture medium for a considerable period and some degradation of the virus may have occurred during the interval of cultivation outside the animal body, although this was not previously thought to have occurred.[7] The serum employed was derived very early in the period of serological response from animals not susceptible to the infectious agent, and such serum may not be comparable to that of animals of a susceptible species following recovery from infection. The question of heterophile reactions between the tissues of the different species employed in the experiments does not seem to apply to the results.

The conclusion appears warranted that the first and probably most specific serological response of rabbits to intraperitoneal injections of tissue culture strains of human or swine influenza virus yields a serum which is essentially specific for the homologous strain, and that these differences in the immune response reflect differences in the antigenic structures of the PR8 and Phila strains of human influenza virus.

---

[6] Alexander, R. A., *Onderstepoort J. Vet. Sci. and Animal Ind.*, 1935, **4**, 349.

[7] Magill, T. P., and Francis, T., Jr., *J. Exp. Med.*, 1936, **63**, 803.

9019 C

Active Anaphylaxis Produced with Specific Carbohydrate of Pneumococcus Type I.

T. J. Kurotchkin and B. F. Chow.

From the Departments of Bacteriology and Immunology, and Biochemistry, Peiping Union Medical College, Peiping, China.

Avery and Tillett[1] have shown that anaphylactic shock may regularly be produced in guinea pigs passively sensitized with rabbit antipneumococcus serum and subsequently injected with specific carbohydrate derived from a homologous microörganism. No parallel positive experiments on the production of active anaphylaxis in guinea pigs have been reported. Previously reported investigation[2] indicated that active sensitization of guinea pigs can easily be brought about, using different gram positive microörganisms. Inasmuch as pneumococcus is also a gram positive organism, active sensitization with cultures of this organism should be positive.

The present experiment records a positive result obtained in the active sensitization of guinea pigs with cultures of pneumococcus type I.

A series of 10 young guinea pigs, weighing from 250 to 300 gm. received repeated intraperitoneal injections of heat-killed blood agar culture of pneumococcus type I grown at an incubator temperature for 24 hours. Each animal received 3 injections given at weekly intervals. The dose administered to each animal contained the total amount of the organism grown upon one blood agar slant. Three weeks later all animals were tested for hypersensitivity through the intravenous injection of the type-specific carbohydrate given in doses from one to 4 mg. The carbohydrate used in this experiment gave negative biuret, xanthoproteic and Millon reactions. When dissolved in ordinary normal saline, traces of it remained insoluble. It was completely soluble in saline adjusted to pH 7.6. In our experiment ordinary normal saline has been used. The traces of insoluble material were removed by spontaneous sedimentation.

Out of 10 guinea pigs treated as described, 6 remained normal, 4 (receiving 2 mg. of the carbohydrate each) showed only a slight embarrassment of breathing. This failure to obtain a positive

1 Avery, O. T., and Tillett, W. S., *J. Exp. Med.*, 1929, **49**, 251.

2 Lim, C. E., and Kurotchkin, T. J., *Far East. Assn. Trop. Med.*, 1934, Trans. **1**, 83.

result suggested an insufficient sensitizing dose and too short incubation time.

Twelve guinea pigs were sensitized by the method previously described. The sensitizing dose of pneumococcus culture was equal to the amount of organisms grown upon 2 blood agar slants in 24 hours' incubation. Four weeks later the anaphylactic test was performed and the result presented in Table I was obtained.

TABLE I.
Showing Active Sensitization of Guinea Pigs with Pneumococcus Type I Cultures.

No. of animals	Dose of specific carbohydrate	Results
	mg.	
2	2	No response
3	4	Dead in 3-5 minutes
2	4	Moderate shock
3	6	Very slight shock
2	8	No response

From this table it follows that altogether 5 animals developed typical anaphylaxis. Three of these animals died in 3-5 minutes while 2, after having developed a moderate anaphylactic shock, recovered. The autopsies performed on the dead animals revealed typical distension of the lungs. As seen from the table, the effective dose of the carbohydrate was found to be equal to 4 mg. Doses smaller or larger than this produced either very slight or no effect. Our experiment suggests that sensitization of guinea pigs with heat-killed cultures of pneumococcus type I requires comparatively large doses of the microörganism and proper adjustment of the dose of the specific carbohydrate.

9020 C

Growth of Cancerous and of Embryonic Tissues Stratified in the Ultra-Centrifuge.*

M. F. GUYER AND PEARL E. CLAUS.

From the Department of Zoology, University of Wisconsin.

In an attempt to upset the normal conditions of cells and thus possibly induce abnormal growths which might give some insight into malignancy the authors subjected various bits of embryonic

* These investigations were supported in part by a Brittingham research grant and in part by a grant from the Wisconsin Alumni Research Foundation.

tissues to intense centrifugalization and implanted them in young rats of 65-90 gm. weight. Small pieces of rat carcinoma were similarly centrifuged and implanted. A Beams air-driven ultracentrifuge, rotated at a speed which produced a displacement pull of about 400,000 times that of gravity, was employed. The embryonic tissue consisted of small bits snipped from the body-wall of living embryos about 2 weeks old. It was placed immediately in isotonic Locke's solution. Half of each bit was transferred to the metal rotor of the centrifuge and the other half implanted subcutaneously into a control rat. Microscopical inspection showed that the cell-contents of the various tissues used had been stratified in the centrifuge in practically the same way as that previously reported for pituitary gland.[1] Representative results which followed implantation are shown in the following paragraphs.

Cancer. In a preliminary test, cancer tissue (Flexner Jobling carcinoma) centrifuged for 10 minutes was implanted in 2 young rats, and similar cancer tissue centrifuged for 20 minutes was implanted in 2 other young rats. Inasmuch as the tissue centrifuged for 10 minutes grew week by week, during 9 weeks of observation, at practically the same rate as non-centrifuged control implants, it was evident that such tissue could withstand the treatment unimpaired. The cancer tissue centrifuged for 20 minutes did not start to grow in one of the host rats; in the other it grew slowly for 6 weeks then regressed from a diameter of 9 mm. to 5 mm. during the next 3 weeks.

As a result of these exploratory tests it was decided to try centrifugalization for about 15 minutes with a sufficiently large number of transplants to give significant results. Accordingly bits of carcinoma measuring about 1 mm. in diameter were centrifuged for 15 minutes and then implanted into each of 10 young rats weighing from 70-90 gm. Of the 10 implants one did not "take". The remaining 9 were observed and measured week by week. All started active growth but by the end of 5 weeks 2 were regressing and the other seven were growing at a rate indistinguishable from that of the controls.

Since 15 minutes of centrifuging produced no perceptible change in the implants, a second series of transplants was made, using cancer tissue centrifuged for 20 minutes. Such centrifuged tissue together with corresponding bits which had been kept in Locke's solution for the same length of time were implanted in 10 young rats (65-90 gm.), a centrifuged piece and a non-centrifuged con-

[1] Guyer, M. F., and Claus, P. E., *Biol. Bull.*, 1936, in press.

trol in each rat. The transplants grew in each of the 10 hosts. In one individual both the centrifuged and the non-centrifuged cancerous growths began to diminish in size after the ninth week and 4 weeks later both areas were healed and the rat free of all cancerous tissue. In the 9 other individuals the growth of each implant was continuous with no significant differences between the experimental and the control tumor, hence the experiment was terminated at the end of 12 weeks.

It is of interest to note that the carcinomatous tissue in question showed less indication of stratification than any tissue we have subjected to prolonged centrifugalization. After 20-30 minutes of such treatment the nucleus though commonly showing some evidence of stratification within itself, underwent little or no dislocation in the cytoplasm, and the cytoplasm seemed little disturbed. Even the Golgi apparatus which exists in a more or less diffuse condition in such cancer cells, largely retained its ordinary distribution. Such evidence would seem to indicate that the cytoplasm of the carcinoma cells in question was physically in the condition of a very stiff gel.

Trophoblast. Trophoblastic tissue removed during early pregnancy and centrifuged for 12 minutes did not grow when implanted into each of 5 young rats. However, similar tissue, not centrifuged, likewise did not grow when implanted into 4 control rats, hence the lack of viability can not be attributed to the centrifugalization.

Body-wall. Small pieces about 2 mm. in diameter, snipped from the body-wall of rat embryos between 10 days and 2 weeks old, were subjected to centrifugalization. The thought behind the experiment was that active mitosis would be in progress and that, therefore, as a result of centrifugal displacement irregular cell divisions might be induced and thus lead to abnormal tissue developments. Similar pieces of tissue were kept in Locke's solution for the same length of time the centrifuged bits were out of the body; they were then implanted as controls in young rats of the same age as those implanted with the centrifuged tissue.

In the first experiment 10 young rats (65-90 gm. in weight) were implanted subcutaneously with bits of embryonic body-wall which had been centrifuged for 12 minutes, and 6 controls were implanted with similar bits of uncentrifuged tissue. Microscopical inspection showed that the centrifuged cells had been much flattened and stratification of cell contents was evident, with the nucleus displaced to the centrifugal side of the cell. The centrifuged tissue resumed growth in 9 of the 10 hosts to which it was transferred, and the non-centrifuged in 5 of the 6 controls. The growth, indicated ex-

ternally by elevations of the skin, was observed and measured week by week. The tissue was removed at the end of 12 weeks from 3 of the hosts, and at the end of 32 weeks from 5 others, and sectioned for study. In one of the 9 animals in which the centrifuged tissue grew, growth ceased at the end of 4 weeks and by the end of 8 weeks the transplant had wholly disappeared. In the other 8 growth continued and reached its maximum in about 8 weeks after which the transplants maintained themselves at the maximal size until removed for examination. The subcutaneous masses, measuring 1.7x1.2, 1.5x1.0, and 1.2x0.7 cm. respectively, removed at the end of 12 weeks proved to be rounded capsules filled with hair. Similarly 2 of the subcutaneous growth masses removed at the end of 32 weeks, measuring 0.5x0.5 and 1.8x1.2 cm. respectively, contained capsules filled with hair. Evidently, although neither hair nor well developed skin were present in the small bit of implanted tissue, less than 2 mm. in diameter, these structures were already determined *in potentia* and despite the violent treatments in the centrifuge the tissue continued to increase in mass and eventually developed into skin bearing hair follicles and hair that seemed in no wise different from that of normal rat skin, except the hair was somewhat longer. That the centrifuging had nothing to do with the phenomenon is evident from the fact that similar capsules containing skin and hair masses developed in 3 of the controls. In one of the rats bearing a centrifuged bit of embryonic body-wall, at the end of 32 weeks the transplant had developed into a flat piece of cartilage and bone measuring 1.3x0.7 cm. Histologically (Fig. 1) the tissue did not look different from that of an ordinary rib undergoing ossification although it was much larger than any normal rib of its host. Presumably a bit of what would have eventually been rib cartilage or bone was present in the centrifuged material and it continued its inherent course of development. The obviously abnormal thing about it was its large size, due probably to a lack of the mechanical and other restraining factors that prevail when such a tissue grows in its normal position as a rib.

In a later experiment a similar growth of cartilage and bone developed from a centrifuged bit of body-wall. In this experiment 5 host rats were used and the bits of tissue from the body-wall were centrifuged 20 minutes before subcutaneous implantation. The rate and manner of growth of this transplanted tissue did not differ noticeably from that of the earlier experiment in which the tissues were centrifuged for 12 minutes. In 2 of the 5 hosts the transplants persisted for about 12 weeks and then disappeared. In 2 of the others encapsulated growths filled with hair were found when the

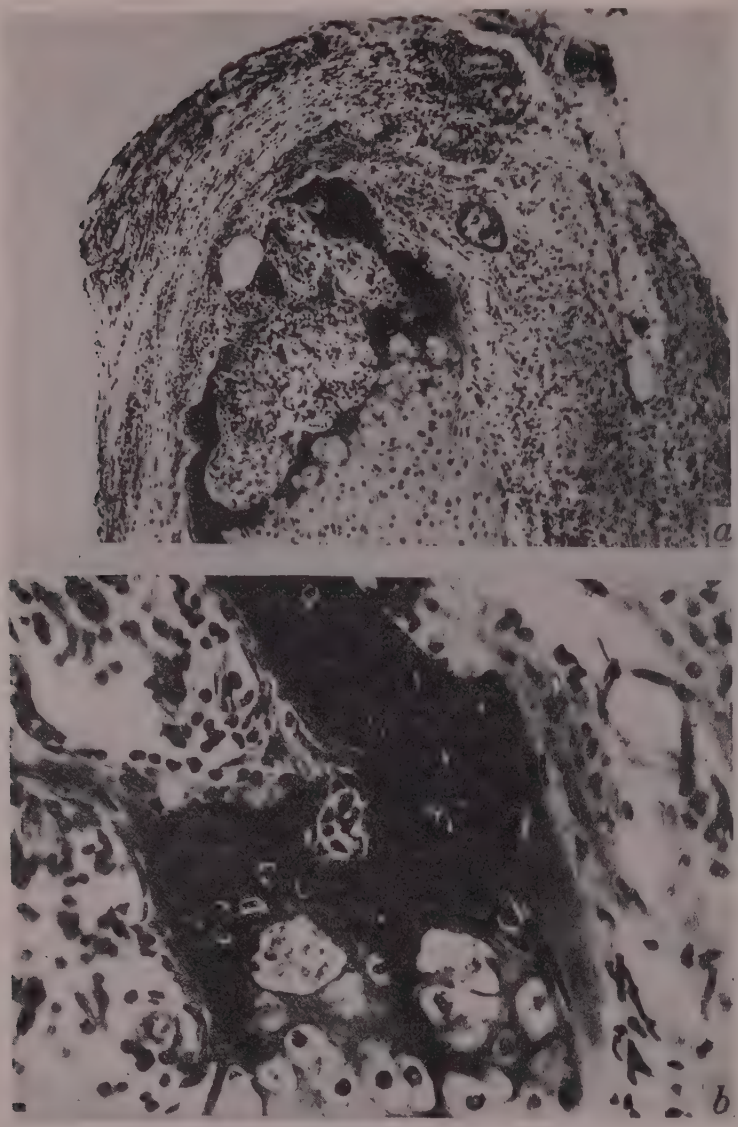


FIG. 1.

Section through part of a mass of cartilage and bone which developed in a rat from a bit of centrifuged embryonic body-wall: *a* × 100; *b* × 970. Ossified areas appear black; the cartilage cells are obvious.

transplants were removed at the end of 12 weeks. In the remaining host the centrifuged bit, as already stated, had grown into a flat piece of cartilage and bone which measured 1.5 by 0.7 cm.

The most significant result observed from the foregoing experiments was the persistence with which cells distorted by violent centrifugalization regained and maintained their usual characteristics. The abnormalities which appeared are probably interpretable as normal tissues developing in unusual locations rather than as the result of fundamental changes induced in the constituent cells by centrifugalization. The host seemed to be merely a nutritional matrix for the centrifuged tissue which developed along the path of its original constitutional trend.

Summary. The cells of carcinoma tissue, after 30 minutes of centrifugalization in an ultracentrifuge at a displacement pull of 400,000 times that of gravity, show little trace of stratification of contents. Apparently the cytoplasm of such cells is of the consistency of a stiff gel. Such cells centrifuged for 20 minutes grew as readily as non-centrifuged cancer cells when implanted in young rats. The cells in bits of embryonic body wall centrifuged for 12 and for 20 minutes respectively, although having their contents markedly stratified by the treatment, resumed growth when implanted subcutaneously in young rats, developing usually into hair-filled cysts of skin but occasionally into cartilage and bone. These results were probably due to the misplacement of embryonic tissues rather than to changes induced by centrifugalization.

9021 C

Retarded and Prolonged Action of Insulin Precipitated by Safranin.

H. R. JACOBS AND H. T. RICKETTS.

From the Department of Medicine, University of Chicago.

The injection into man and animals of a suspension of insulin precipitated by a protamine has been shown to lower the blood sugar for a period several times as long as that given by ordinary insulin.[1] The appearance of an article by Walker[2] on the use of dyes to pre-

[1] Hagedorn, H. C., Jensen, B. N., Krarup, N. B., and Wodstrup, I., *J. Am. Med. Assn.*, 1936, **106**, 177; Root, H. F., White, P., Marble, A., and Stotz, E. H., *J. Am. Med. Assn.*, 1936, **106**, 180.

[2] Walker, A. W., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 726.

cipitate proteolytic enzymes and bacteriophage led to the idea that such precipitants might be effective, when added to commercial insulin, in producing a suspension which would behave like protamine insulin.

Accordingly, a preliminary experiment was undertaken using equal parts of (1) U-40 insulin and (2) a 0.5% water solution of safranin O. After standing for 2 days at room temperature the mixture yielded a small amount of granular precipitate. The addition of one drop of 0.1 N NaOH produced an immediate flocculation which subsequently disintegrated into finer particles, leaving the solution turbid. By means of a series of phosphate buffer mixtures[3] it was found that precipitation occurred almost equally well at all pH values between 7.0 and 8.0. Since, however, insulin decomposes rapidly in alkaline solutions, all subsequent precipitations were carried out in as nearly neutral a medium as possible—namely, at pH 7.2.

Animal Experiments. The activity of the precipitated material was tried on one dog which was normal except for distemper and on one depancreatized dog.

(1) "Normal" dog, weight approximately 10 kg. The safranin precipitate from 5 cc. of U-40 insulin was washed once with water, added to the precipitates obtained during the experiments with the phosphate buffers, the whole redissolved in 6 cc. of a mixture of 1 volume of 0.1 N HCl and 2 volumes of 1.15 M KH_2PO_4, and injected intravenously after the animal had fasted for 19 hours. Determinations of the blood sugar showed the development of hypoglycemia which persisted for at least 7 hours.

(2) Depancreatized dog, weight 11 kg. Complete pancreatectomy had been performed 8 months previously and the animal had been maintained constantly on a weighed diet, including raw pancreas, and 18 units of insulin twice daily. In this series of experiments it was desired to compare the effects of (a) safranin insulin suspension, (b) the redissolved precipitate of safranin insulin, (c) protamine insulin suspension, (d) the redissolved precipitate of protamine insulin, and (e) regular insulin.

The safranin insulin suspension was prepared by adding 0.5 cc. of U-40 insulin to 0.5 cc. of saturated safranin O solution in a phosphate buffer of pH 7.2. The safranin insulin precipitate was obtained by a similar procedure, following which the mixture was centrifugated, the supernatant fluid poured off and drained, and the

[3] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins Co., Baltimore, 1932, vol. II, p. 816.

residue dissolved in 1 cc. of acid buffer (1 volume of 0.1 N HCl to 2 volumes of 1/15 M KH_2PO_4). The protamine insulin suspension was made according to directions and from materials supplied by Eli Lilly and Company. One cubic centimeter of buffered protamine solution containing a small amount of zinc is added to 4 cc. of U-50 insulin, resulting in 5 cc. of U-40 protamine insulin. One-half cubic centimeter of such a suspension was used for injection. The protamine insulin precipitate was obtained by centrifugating 0.5 cc. of the standard suspension, pouring off the supernatant fluid, wiping the sides of the tube dry and redissolving the residue in 1 cc. of the acid buffer solution described above.

Thus, in each experiment in which the suspension was used, the material to be injected was made up to contain 20 units of insulin. In each case in which the redissolved precipitate was employed, the amount injected was the amount precipitated from 20 units of insulin, and, since the precipitation of insulin is probably incomplete, it presumably contained less than 20 units. In the experiment in which regular insulin was used the dose was 20 units.

At intervals of one week or more one of the test substances was injected subcutaneously after the animal had been deprived of food and insulin for 24 hours. Blood for sugar determination was withdrawn from the external jugular vein immediately before and at intervals of 2 hours after the injection until 12 hours had elapsed. The final sample was taken the following morning before the administration of food and insulin and approximately 24 hours after the injection of the test substance. Analyses of the blood for sugar were performed by the Shaffer-Hartmann method[4] on unlaked blood filtrates made according to Folin.[5]

The results, shown in the accompanying table and chart, indicate that safranin insulin suspension rather closely resembles protamine insulin suspension in its ability to lower the blood sugar gradually and to maintain hypoglycemia for at least 12 hours, though the latter is somewhat more effective in both respects. Since the redissolved precipitates presumably contained less than 20 units of insulin, it is not surprising that the duration of their action was shorter than that of the suspensions; it was also shorter than that of 20 units of regular insulin. The redissolved protamine insulin precipitate reduced the blood sugar at almost exactly the same slow rate as did the safranin insulin suspension up to and including the 6-hour period, beyond which the latter was distinctly more effective in

[4] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1921, **45**, 365.

[5] Folin, O., *J. Biol. Chem.*, 1930, **86**, 173.

TABLE I.

Effect of Subcutaneous Injection of Suspensions, Precipitates and Solution of Insulin on the Blood Sugar of a Depancreatized Dog.

Hours after injection	Blood sugar in mg. per 100 cc. after injection of				
	(a)	(b)	(c)	(d)	(e)
0	361	413	387	324	390
2	143	43	166	141	48
4	21	—	33	21	18
6	26	42	25	26	16
8	19	31	25	27	30
10	26	92	18	54	46
12	37	182	18	129	107
±24	351	334	293	348	—

(a) = 20 units safranin insulin suspension.
(b) = Redissolved precipitate from 20 units insulin precipitated by safranin.
(c) = 20 units protamine insulin suspension.
(d) = Redissolved precipitate from 20 units insulin precipitated by protamine.
(e) = 20 units regular insulin.

maintaining hypoglycemia. The redissolved safranin insulin precipitate, on the other hand, for reasons which are not clear, produced a rapid and brief lowering of the blood sugar, simulating the action of regular insulin..

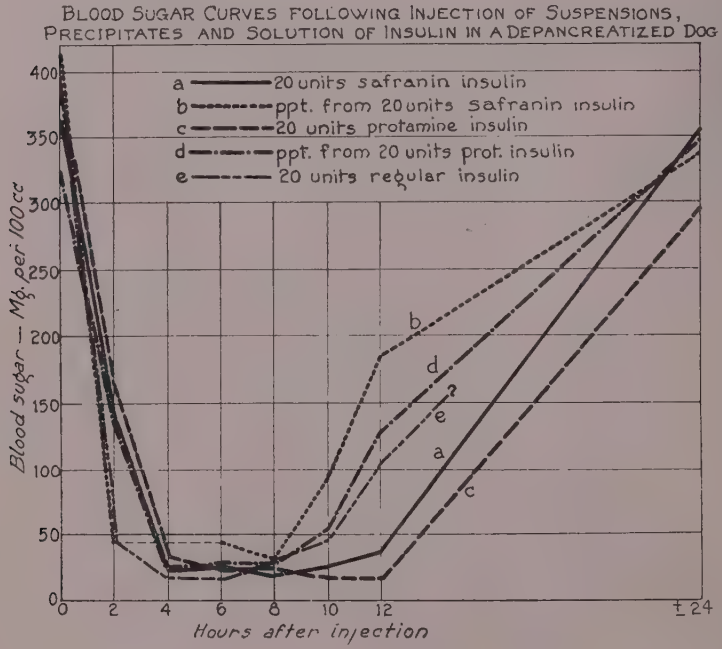


FIG. 1.

Summary. It has been shown that the addition of a weakly alkaline, buffered solution of safranin to a solution of commercial insulin yields an insulin-containing precipitate which, when injected in suspension into animals, causes hypoglycemia of gradual onset and extended duration. The blood sugar curve so produced is similar to, but not quite so depressed as, that given by the injection of an equal amount of protamine insulin suspension. The redissolved precipitates of safranin and protamine insulin are considerably inferior to the suspensions in retarding the fall of the blood sugar and/or prolonging the period of hypoglycemia.

9022 C

Bactericidal Effects of Vapors from Crushed Garlic on *Mycobacterium leprae.*

ROBERT S. MCKNIGHT AND CARL C. LINDEGREN.

From the Department of Bacteriology, College of Letters, Arts and Sciences, University of Southern California, Los Angeles.

Walton, Herbold, and Lindegren[1] showed that the vapors from freshly crushed garlic were germicidal to certain organisms. The present paper reports the effects of these vapors on different strains of acid-fast and non-acid fast *Mycobacterium leprae.*

Petri dishes containing 3% glycerin nutrient agar were warmed in an incubator at 37.5°C. for about 2 hours and a heavy suspension of organisms was then spread on the agar. After inoculation, one gram of garlic, freshly ground in a meat-chopper, was placed on the inverted cover of the petri dish below (but not in contact with) the agar. The dish was sealed with a large rubber band and placed in the incubator at 37.5°C. The fumes from the garlic were allowed to fill the air-space below the agar surface. The amount of volatile substances transported to the agar was varied by exposing the agar to the fumes of the garlic for different lengths of time. Intervals of from one minute to 2 hours were used. At the end of each interval, the dish was removed from the incubator and the cover containing the garlic replaced by a sterile cover. Then the dish was returned to the incubator and after 3 days' incubation the amounts of growth on the various plates were compared (Table I). The heaviest growth was given a score of 4. If the treated plates

[1]Walton, L., Herbold, M., and Lindegren, C. C., *Food Research,* 1936, **1**, 163.

TABLE I.

Comparative Amounts of Growth of *Mycobacterium leprae* on Agar Plates Exposed to the Vapors from One Gram of Crushed Garlic at 37.5°C.

Characteristics of Strains	Exposure in minutes								
	0	1	2	4	8	16	32	64	128
Non-acid-fast Yellow	4	4	4	4	2	1	2		
	4	4	4	4	2	1	1		
	4	4	4	4	3	2	1		
	—	4	4	4	3	1	1		
	4		4	—	—		1	0	
	4		—	3	3		2	1	
	4		4	4	—		1	0	
	4		4	4	4		2	0	
	4		4	4	—	0	0	0	0
	4		4	3	2	1	0	0	0
	4		4	2	1	0	0	0	0
Non-acid-fast Orange	4	4	3	3	3	2	0		
	4	4	4	3	3	2	0		
	4		—		2		1	0	
	4		4		—		2	1	
	4		3		3		1	0	
	4		3		3		1	0	
Acid-fast Yellow	4	4	4	4	4	2	2	0	
	4	4	4	4	3	3	1	0	
	4	—	4	4	4	3	1	0	
	0		4	3	1	0	0	0	0
	0		4	4	2	2	1	0	0
	0		4	4	2	0	0	0	0
	4		4	4	4	3	1	—	
	4		4	4	4	3	2	0	
	4		4	4	4	—	2	0	

showed about three-fourths of this amount, they were scored 3; if about one-half, they were scored 2; if about one-fourth, they were scored 1; if no growth occurred, they were scored 0.

A non-acid-fast yellow strain of *M. leprae* obtained from Dr. T. D. Beckwith, a non-acid-fast orange strain from Dr. J. F. Kessel, and an acid-fast yellow strain from the American Type Culture Collection were used. As indicated in Table I, the tests were run in 8 batches. In each batch, sets of either 3 or 4 plates were treated at each of the different time exposures. Each row across the page indicates the scores of one of the sets of plates in each batch. The control plates were usually spread last. In one batch using the acid-fast yellow strain, it is clear that spreading of the control plates was forgotten. A blank space indicates that no test was made. A dash indicates that a contamination made it impossible to estimate the amount of growth accurately.

On plates exposed for 32 minutes, growth was markedly inhibited and practically no growth occurred on plates exposed for an hour or more.

The writers are indebted to Mr. Ernest C. Phillips for performing many preliminary experiments which were consistent with those herewith described.

9023 C

Body Temperature and Plasma Lipids in Rabbits.*

ELDON M. BOYD, J. H. ORR AND G. B. REED.

From the Departments of Pharmacology and of Bacteriology, Queen's University, Kingston, Ontario, Canada.

Chauffard, Laroche and Grigaut demonstrated that there was an inverse relationship between body temperature and serum cholesterol in patients afflicted with typhus. This observation has been repeatedly confirmed in practically all of the commoner febrile conditions of man and of some animals. Boyd,[4] in whose paper a comprehensive bibliography appears, showed that not only did febrile temperatures affect plasma cholesterol but also the concentration of other lipids in both plasma and in the red blood cells. Similar data were simultaneously published by Stoesser and McQuarrie.[7, 8, 9] This work has proven that during an acute febrile condition there occurs a lipopenia (term introduced by Boyd[4]) or decreased concentration of blood lipids and that the decrease occurs in plasma and not in the red blood cells in which latter the lipid values may actually increase.

The present paper is concerned with further research into the significance of this febrile lipopenia and in particular represents an attempt to evaluate the effect of temperature *per se*. McQuarrie and Stoesser[6] reported that no change occurred in the value of

* This work was aided financially by the Alice F. Richardson Fund of the Kingston General Hospital. The authors wish to thank Messrs. J. W. Stephenson and E. A. Watkinson for technical assistance.

4 Boyd, E. M., *Canadian Med. Assn. J.*, 1935, **32**, 500.

7 Stoesser, A. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1324.

8 Stoesser, A. V., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1326.

9 Stoesser, A. V., and McQuarrie, I., *Am. J. Dis. Child.*, 1935, **49**, 658.

6 McQuarrie, I., and Stoesser, A. V., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1281.

serum cholesterol in one patient given an artificial fever by the use of phenylethylhydantoin nor in a second case of diathermy. They[9] recorded that in 9 subjects given artificial fever by diathermy, phenylethylhydantoin and foreign protein the pyrexia did not affect either plasma cholesterol, "lecithin," or total fatty acids. They concluded that a rise in body temperature is not of itself sufficient to produce a lipopenia. In the present investigation variations in temperature of normal animals, variations not produced artificially or by interfering in any way with the normal metabolism of the animals, were compared with plasma lipid values.

The young rabbit proved a suitable species for this purpose. A colony of 30 animals, mostly males and averaging about 6 months old, was obtained from the farms and housed under supervised standard conditions in the animal house. After 1 to 2 months under these conditions, examination revealed a considerable variation in rectal temperature, a variation between 100 and 103°F., among the animals which strict examination revealed to be otherwise in apparent excellent health.

Blood was obtained by cardiac puncture under aseptic conditions and with the use of a mild ether anesthesia (not over 5 minutes of anesthetic in any case) after having fasted the animals over night. The blood was heparinized using 1 mg. of heparin (Connaught Laboratories) per 10 cc. of blood and immediately centrifuged to obtain the plasma. For the purposes of the present study it was deemed sufficient to select 2 lipids as examples of the plasma lipids and determine any relation between their values and changes in body temperature. The two lipids selected were phospholipid and free cholesterol. Extracts were prepared by adding 3 cc. of the heparinized plasma to 80 cc. of alcohol-ether and filtering without heating, using the principle of cold extraction of lipids developed by Boyd.[5] The resulting extracts were analyzed by Bloor oxidative micromethods as modified by Boyd.[1, 2, 3]

The principle of cold extraction of lipids was worked out by Boyd[5] on human plasma in which it was shown that a dilution of one part of plasma in 20 or more parts of alcohol-ether extracted immediately and without heat all lipids capable of being extracted by this solvent under any conditions. It was decided to prove at the beginning of this study if the same proportions and procedures did or did not hold for rabbit plasma. Extracts were thus prepared

[5] Boyd, E. M., *J. Biol. Chem.*, 1936, **114**, 223.
[1] Boyd, E. M., *J. Biol. Chem.*, 1931, **91**, 1.
[2] Boyd, E. M., *J. Biol. Chem.*, 1933, **101**, 323.
[3] Boyd, E. M., *J. Biol. Chem.*, 1935, **110**, 61.

from the blood plasma of 8 rabbits (a) using Boyd's[5] cold extraction and a dilution of 1 in 25 or better and (b) by boiling such extracts for a period of 15 minutes, adding fresh solvent to replace that lost by evaporation on the steam bath. The phospholipid and free cholesterol of these extracts were determined. It was noted that there was as much or more of these lipids present in the unheated as in the heated extracts in practically all cases. In fact the results suggested that boiling may destroy some of the more delicate phospholipid and hence be undesirable. It may thus be concluded that cold extraction with sufficient dilution is superior to the use of heat in preparing extracts of rabbit plasma, confirming the studies of Boyd[5] on human plasma.

TABLE I.

A Comparison of Values for Plasma Phospholipid and Free Cholesterol with Body (Rectal) Temperature in Normal Young Rabbits.

No. of Animals	Rectal Temperature	Phospholipid (Mg. per 100 cc.) (Mean ± St. Dev.)	Free Cholesterol (Mg. per 100 cc.) (Mean ± St. Dev.)
5	100—100.9°F	60±11	15± 9
10	101—101.9	65±26	12±11
15	102—103	62±29	15±11

In Table I the concentration of plasma phospholipid and free cholesterol of the 30 normal young rabbits have been shown in relation to increasing rectal temperatures of the group. The percentage of plasma phospholipid in the animal with lowest body temperature was 55 mg. % and that in the animal with the highest body temperature was 47 mg. %. Corresponding values for plasma-free cholesterol were 10 and 14 mg. % respectively. These results typify what may be seen from an inspection of Table I, namely, that there was no inverse relationship between the concentration of these 2 plasma lipids and increasing body temperatures in this group of animals. When the results were plotted a similar lack of any relationship was seen.

The rectal temperatures of these animals were followed subsequently to the initial bleeding for a period of some 2 months. Variations were seen to occur in individual animals comparable to the variations in the group. About 6 weeks after the initial bleeding a second blood sample was taken and its plasma phospholipid and free cholesterol estimated and compared with the change in body temperature of each animal which had meanwhile taken place. This second set of analyses was done on 21 of the original 30 animals. In 13 out of 21 an increase in rectal temperature had occurred and in 8 a decrease or no change. In 15 of the 21 animals plasma phos-

pholipid fell in value and in 6 it rose. Plasma-free cholesterol had fallen in 10 and risen in 11 rabbits. A reciprocal change between body temperature and plasma phospholipid was noted in 12 of the 21 and no reciprocal change in 9. Plasma-free cholesterol varied inversely with the recorded change in temperature in 9 of the 21 animals and directly in 12. In other words, there was no consistent reciprocal relation between the concentration of plasma phospholipid and free cholesterol and changes in body temperature in these individual rabbits. These experiments demonstrate thus that there is no relation between variations in the normal body temperature of rabbits and the concentration of plasma phospholipid and free cholesterol. In conjunction with the previous work of Stoesser and McQuarrie[1,2] they indicate that body temperature itself is not the factor responsible for the variations in blood lipids seen in the lipopenia of fever.

Summary. Plasma phospholipid and free cholesterol were estimated by oxidative micromethods in 30 normal young rabbits and their concentration found to bear no relation, individually or collectively, to variations in normal body temperature between 100 and 103°F.

9024 C

Effect of Amputation of Apical Portion of Uterine Horn Upon Labor.

D. N. DANFORTH, R. R. GREENE AND A. C. IVY.

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

Visual inspection of the monkey uterus in labor indicates that the uterine contractions start in the region of insertion of the tubes and then spread over the body of the uterus.[1] One obtains the idea that the region of the tubes serves as a sort of "pace-maker" for the uterus. In the post-partum uterus of the dog many waves of contraction start at the apex of the horns. This suggests that in the dog as well as in the monkey the waves might originate preferentially in the region of the tubes. In the uterus of the pregnant dog the contractions appear to originate usually in the ampulla that is being

[1] Ivy, A. C., Hartman, C. G., and Koff, A., *Am. J. Obst. and Gynec.*, 1931, **22**, 388.

evacuated rather than in the apices of the horns." However, it is possible that the contractions in the active ampulla are due to an "impulse" that originates in the apex of the horn and does not cause contraction until it reaches the ampulla that is ready for evacuation. This work was undertaken to ascertain whether or not the apex of the horn is essential for normal parturition.

Three dogs, 5 rats and 2 rabbits were used in this study. Shortly before the expected date of delivery they were operated upon under ether anesthesia. In each case the distal fetus of one horn was gently milked toward the corpus of the uterus in order that a ligature might be placed about the apical portion of the horn. A figure of eight suture-ligature was then applied, and directly distal to this a second ligature was placed. The horn was then severed between them. In all of the rats and in one rabbit the apical portion of the cut horn was removed from the abdomen. In the other animals it was left in place.

All of the animals so treated showed no impairment of the processes of labor. Labor did not appear to be prolonged, and all of the fetuses were delivered with no apparent embarrassment on the part of the mother. Autopsy at a later date showed normal involution of both horns, the control horn appearing to differ in no way from the horn of which the apical portion had been amputated.

Rudolph and Ivy[2] showed that in the dog separation of the uterine horns from the corpus at their origin from this structure interfered only temporarily with coördinated contractions of the post partum uterus. In order to disturb the coördinated nature of uterine contractions between the corpus uteri and the horns it was necessary to separate the horn from the corpus and also excise the utero vaginal ganglion. Coördinated activity occurred when the utero vaginal ganglion alone was excised. This showed that the coördination of uterine contractions could be maintained by either an intrinsic mechanism, or by an extrinsic mechanism in the ganglion. They further found that excision of the ganglion did not materially alter parturition insofar as the uterus was concerned. However, in their work they did not rule out the rôle that the apical portion of the horn might play in the coördinated activity of the horn itself. Our results together with those cited above indicate that each ampulla in the dog, rabbit and rat is automatic in regard to the contractions necessary for the evacuation of the uterus. However, to prove this conclusively both the ganglion and the apical portion of the horn would have to be excised in the same animal.

[2] Rudolph, L., and Ivy, A. C., *Am. J. Obst. and Gynec.*, 1930, **19**, 317.

[3] Rudolph, L., and Ivy, A. C., *Am. J. Obst. and Gynec.*, 1931, **21**, 65.

Conclusion. The apical portion of the uterine horn is not essential for the evacuation of the uterus in the dog, rabbit and rat.

9025 C

Effect of Gonadotropic and Oestrogenic Hormones on Regenerating Feathers of Weaver Finches (*Pyromelana franciscana*).*

EMIL WITSCHI.

From the Zoological Laboratory, State University of Iowa.

The seasonal change of plumages in certain birds has long attracted the attention of ornithologists though until recent years little has been accomplished in the analysis of factors in control of this periodicity. The literature shall be considered in a following paper, which will deal with the many correlated features of seasonal variation in different species. The present paper reports only some experiments on the effects of certain hormones on the breast feathers of the African orange weaver finch (*Pyromelana franciscana*). The females carry a hen plumage throughout the year. The breast appears white in the midventral region, as in Fig. 1, left breast, turning into a light buff laterally. The base of the feathers, always invisible in the well arranged plumage, is of a dark gray, due to the deposit of melanin granules (Fig. 3, top left). The males also have this hen plumage outside of the breeding season. It is identical with the plumage of the female to such an extent that it is impossible to tell the sexes apart, unless an occasional nuptial feather may be found that was not shed during the preceding molt. With the approach of the breeding season the males pass through an incomplete molt, shedding only the "small plumage" which is replaced by a brilliant cock plumage in orange-red and black colors. The breast feathers are black (Fig. 1, right breast). Along with the color, the size and shape of the feathers also change (Fig. 4, lower row, right). Regenerating feathers assume the cock type during the active breeding season (about June 15 to September 30) and the hen type during the remainder of the year. Castrated males and females show in principle the same seasonal periodicity as normal

* Aided by grants from the National Research Council, Committee for Research in Problems of Sex. The author is indebted to Dr. O. Kamm for some of the gonadotropic preparations, to Dr. F. C. Koch for the male sex hormone, and to Dr. E. A. Doisy for the female sex hormones used in these experiments.

males (Witschi[1]) though molting and regeneration are more irregular and the plumage of castrates outside the breeding season is sometimes of an intermediate type.

FIG. 1.

FIG. 2.

Castrated male (OW86) in the quiescent season. Hen plumage except for the right throat, breast and abdomen, where under the influence of injections of gonadotropic hormone (preg. mare serum, 20 DRU, 16 days) a cock plumage has regenerated.

FIG. 2.

Castrated male (OW91) with areas 1 and 2 on breast showing regeneration of feathers under conditions described in text.

The cock plumage, obviously is not determined by testicular hormone, since castration does not prevent its appearance during the breeding season. Indeed it has been found that injection of various doses of male sex hormones does not interfere with regeneration of hen feathers during the quiescent period (6 cases). On the other hand, the cock plumage can not be considered as the neutral phase, since it is replaced by the hen plumage after the breeding season.

Continuance of periodicity in castrates suggested that the cock plumage appears in direct response to a high level of gonadotropic hormones. Hypophyseal extracts and serum of pregnant mares were, therefore, injected in 15 different cases (3 males, 1 female, 9 male castrates, 2 female castrates) during the season of sexual quiescence. Feathers regenerating under the influence of these hormones were of the cock type (Fig. 1, right breast). Hypophyseal extracts were administered at the rate of one to 2 rat units

[1] Witschi, E., *Wilson Bull.*, 1935, **47**, 177.

per day. The change to the cock type was only incomplete, while with this dosage ovaries and testes showed very marked stimulation. The pregnant mare serum containing about 20 daily rat units caused a complete reversal to the cock type in all injected males (2) and castrates (8). A study of the effect of different fractions of hypophyseal extracts is now under way.

In bird OW91 (Fig. 2) feathers of the breast region were plucked at intervals of 4 days. The first area, posterior left breast, was deplumed 8 days before starting the injections; the second area, anterior left, 4 days before injections; the third area, posterior right (not visible on the figure), was deplumed on the day when the first injection was given. Injections of pregnant mare serum (20 DRU) were given over a period of 8 days. On the third day the darkening color of the feather papillae indicated melanin

FIG. 3.

Single feathers of castrate OW91 from area 1 (top row), area 2 (middle row) and area 3 (bottom row). Feathers at the left ends of the top and bottom rows are of the pure hen type, grown before and after the period of injections. See text.

production. The photograph (Fig. 2) was taken a week after the last injection. The first deplumed area appears mottled, the second area black and the third, not visible area is white with black dots. Plucked feathers, as shown in Fig. 3, permit one to reconstruct the course of feather regeneration quite completely. After removal of a feather it takes at least 3 days for the feather germ to reorganize and to form a new papilla. In the first area, feathers, therefore, have a growth period of about 4 days before the beginning of injections. During this period, the tip of the feather forms according to the hen type (4 last feathers in top row; the first one represents the hen type of the feathers which were removed from this bird). In the second area active growth starts only in few feathers prior to the injections. Therefore,we find that in this region the feathers have black tips. The tips of these feathers are blunt as is characteristic of the cock plumage (Fig. 3, middle row). During the following 8 days feathers in both areas grow "cock-type". Shortly after the last injection they resume, however, the hen type mode of differentiation, so that the base of nearly all feathers is again in conformance with the season. Quite interesting is also the reaction observed in the third area. The start of regeneration is obviously retarded due

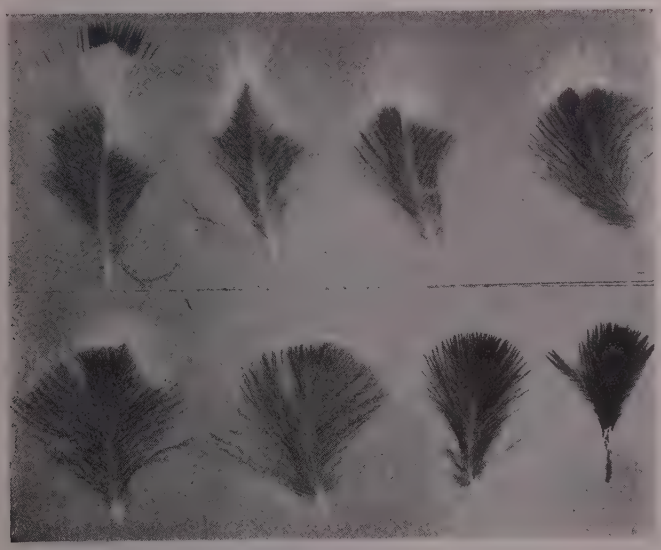


FIG. 4.

Single feathers from the breast of male OW100, grown during the breeding season and showing the effect of a 6-day period of injections of theelol (see text). The last 2 feathers are of the pure cock type, regenerated after injections had been discontinued.

to the disturbance caused by the daily injections. Only the tips of the feathers show, therefore, the full effect of the injections (Fig. 3, bottom row). Some seem to have grown for 2 or 3 days under gonadotropic control (feathers at right end of the row); others show only slight modifications at the very tip, and many have grown entirely after the injections had been discontinued. Thus the feather at the left end of the bottom row (Fig. 3) shows that differentiation reverts to the hen type soon after the last injection.

The realization of the rôle of the gonadotropic hormone in determining the cock plumage raises the question why the female retains hen feathers during the breeding season. No doubt the gonadotropic hormone reaches also a high level in females, as indicated by the growth and ovulation of eggs and the plumage change in castrated females. Injection experiments prove that in Pyromelana as in the domestic fowl the cock feathering is inhibited by ovarial hormones. Three males and 5 castrates which had started to assume their breeding plumages were used in this experiment. Injections were given twice daily for a period of 6 days. The crystalline hormones were dissolved in weak alcohol. One group was given 20 RU (10γ) per day of theelin, the second group was given 20 RU (1.25γ) per day of dihydro-theelin and the third group obtained 4 RU (40γ) per day of theelol. In every case feathers regenerating during these 6 days differentiated according to the hen type instead of the cock type, as would correspond to the season. Fig. 4 shows some feathers of the male OW100, of the group which obtained theelol injections. The first feather in the upper row had started to regenerate shortly before the injections; its tip is black but it is followed by the parts formed under the influence of theelol, which are of the hen type. The following 5 feathers started under the influence of the injections; the tip is henny, but the lower parts reverted to the cock type after discontinuation of the injections. The width of the light edge indicates to what extent regeneration took place within the 6-day period of injections. The last 2 feathers regenerated entirely after this period. They represent again the pure cock type. It is interesting to note that the reversion to the structure of the cock feather precedes distinctly the appearance of the characteristic melanin coloration.

These experiments lead to the conclusion that (1) the hen plumage represents a neutral, basic condition; (2) the cock plumage is induced by a high level of gonadotropic hormone, and (3) the ovarial hormones offset the inductive effects of gonadotropic hormones on the feather type. It is a possibility worth experimental investigation, that the same mode of control may determine feather

patterns in the domestic fowl (brown leghorn), except that hypophyseal activity does not seem to suffer such pronounced seasonal variations as in Pyromelana.

9026 P

Effect of Thyro-Parathyroidectomy in New Born Rats.*

THEODORA NUSSMANN SALMON. (Introduced by Earl T. Engle.)
From the Department of Anatomy, College of Physicians and Surgeons, Columbia University.

Rats are particularly adapted for experiments on the effect of very early ablation of the thyroid because they are very immature at birth, and have had a developed thyroid only the last 3 days *in utero*.[1] No previous reports on mammals have dealt with such early ablations. The present experiments deal with the removal of the thyro-parathyroid apparatus in young rats on the first or second day of life. A very few animals were also operated on the seventh and fourteenth days of life. Complete removal is extremely difficult because of the gelatinous consistency of the infantile tissues. Every animal here reported was checked for completeness of removal by microscopic examination of serial sections of the tracheal region made at autopsy. This check was found to be absolutely necessary since in many stunted animals microscopic remnants were found. These will be treated separately as "incompletes."

Of 486 rats thyro-parathyroidectomized on the first or second day of life, 167 survived the critical period of the first post-operative week. Animals were sacrificed at various intervals after data on growth had been secured. The 167 survivors were autopsied and 107 of them have been examined for completeness of removal. Twenty of these show no remnant of the thyro-parathyroid apparatus. Of the 46 animals operated on at 7 or 14 days, verifying examination of the operative field had been completed on only 5. In an operated control series of 38 animals, the thyro-parathyroids were removed on the first day and the glands re-implanted in the same animal[2]; 30 survived beyond the critical week: 28 showed normal

* Aided by a grant from the National Research Council Committee for Research on Problems of Sex, administered by Dr. P. E. Smith.

1 Kull, Harry A., *Anat. Rec.*, 1926, **32**, 133.

2 Salmon, T. N., and Severinghaus, A., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 251.

growth curves. Some of them have not yet been sacrificed for examination. These animals show that the operation itself is not a factor in causing a retardation of development.

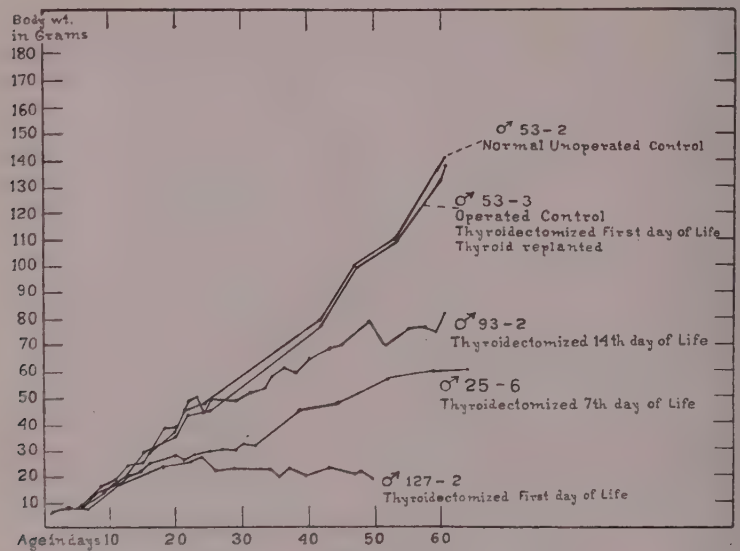


FIG. 1.

Fig. 1 shows the difference in growth response when the thyroid is removed on the first, seventh, and fourteenth days of life. A single animal is allowed to represent its type, since the various animals in each group follow almost exactly the same curve. There is no essential difference between males and females except that after 40 days the weight of the normal males exceeds that of the normal females. All animals completely thyroidectomized on the first or second day of life stop gaining in weight when they attain 20-25 gm. and remain at approximately this weight for the rest of their lives, which are not prolonged beyond 50-60 days because of intercurrent infection.

Only the animals operated on the first or second day of life have symptoms resembling those of human cretinism; including lowered body temperature, sluggish reflexes, awkward muscular movements, lack of response to startling noises (possibly deafness), susceptibility to infection, persistence of infantile skull proportions, and greatly retarded skeletal development. After the early cessation of growth shown by these animals, all anatomical advancement seems to remain at a standstill, suggesting that even if they could be kept alive

beyond 50 to 60 days no further differentiation could be expected from them. They were subject to violent attacks of tetany, which occurred spontaneously or could be induced by ether anesthesia. This made taking measurements of body and tail length at frequent intervals impractical.

The testis weights of the thyroidectomized animals were far below normal, but microscopically the differentiation in experimentals and controls was similar up until the 23rd day, at which time spermatocytes appeared in the periphery of the tubules and a few large degenerating sells were seen in the center. Animals operated on the first or second day of life have not been found to progress beyond this stage, the microscopic appearance of their testes being unchanged at the age of 50 days; whereas the controls by that time show complete spermatogenesis.

In the female the course of follicular development at first resembles that of the normal, but in the experimentals development was arrested after partial antrum formation (14 days).

The thymus was extremely diminished in size, consisting of but a few shreds of tissue. A qualitative examination of the adrenals indicates a reduction of cortical tissue.

9027 P

Action of Merthiolate on Gonadotropic Effect of Anterior Pituitary Extract.

GRAHAM CHEN AND H. B. VAN DYKE.

From the Department of Pharmacology, Peiping Union Medical College.

The germicide, "Merthiolate"* (C_2H_5HgS C_6H_4COONa), should be included in the growing list of substances which may enhance the gonadotropic effect of anterior pituitary extract. In using merthiolate as a germicide in aqueous solutions of gonadotropic pituitary hormone, we discovered by accident that the gonadotropic changes were markedly potentiated because of the presence of low concentrations of the drug.

If a dose of anterior pituitary hormone in aqueous solution at a pH of about 7.4 is administered by subcutaneous injections distributed over several days to immature female rats and causes a

* The merthiolate was furnished us through the courtesy of Prof. M. S. Kharasch and the Eli Lilly Co.

moderate but clear-cut increase in ovarian weight, the same solution containing 1-5000 merthiolate (total dose 0.8 mg.) causes twice to three times as great a change as judged by the increase in ovarian weight. The merthiolate is added each day just before injection; it causes no change in the appearance of the water-clear hormone solution. An increase in the effect, roughly proportional to the change in concentration, is produced by increasing the concentration of merthiolate. Maximal potentiation occurs in immature rats receiving toxic but sublethal doses of merthiolate (0.077% or 1-1300; total dose 3.08 mg.). Concentrations of 1-10,000 or lower are without effect.

The effect of anterior pituitary extract on the testis of the immature male rat (change in seminal-vesicle weight) is also clearly potentiated by merthiolate. However, the germicide increases neither the proportion of rabbits ovulating nor the number of ruptured follicles in individual rabbits if the effects of the intravenous injection of a solution of an anterior-pituitary extract with and without merthiolate (1-5000 or 1-3000) are compared. Moreover, it does not increase the effect of even crude preparations of prolan on the ovary or testis (seminal vesicles) of the immature rat.

The potentiating effect of merthiolate appears not to be due to any action on the immature animal's own pituitary, for (1) the administration of the germicide alone has no gonadotropic effect, (2) the germicide is without action if it and anterior pituitary extract are administered simultaneously but in different subcutaneous sites, and (3) the potentiating effect is absent if the merthiolate-hormone solution is administered intraperitoneally. Possibly the drug alters the rate of absorption of the hormone (the tissues about the injection-sites appear fibrosed) although other changes under investigation may be important.

The conclusions outlined in the foregoing account are based on experiments in more than 300 immature rats and 70 rabbits.

9028 P

Time Factor Relationship of Follicle Stimulation and Luteinization in the Immature Rat.

ROBERT T. FRANK AND UDALL J. SALMON.*

From the Department of Gynecology and Laboratories, Mount Sinai Hospital, New York City.

In a previous communication from this laboratory it was shown that both luteinizing and follicle stimulating reactions can be obtained with extracts prepared by the acetone precipitation method[1] from castrate and post-menopause urines. It was also demonstrated that with the same extract either pure follicle stimulation or luteinization or combinations of both effects may be obtained by varying the dosage of the extract injected.[2] In these studies all the animals were sacrificed 96 hours after the first injection.

The present investigation was undertaken to determine the effect upon the ovarian reaction produced by varying the time factor.

The gonadotropic material used was obtained from the 4 following sources: (1) castrate urine prepared by the acetone precipitation method, (2) whole pregnancy urine, (3) anterior hypophysis 'Maturity Factor'† (Collip), (4) placenta—'A.P.L.'† (Collip). Litter mate immature female rats 25-30 days old were used in the majority of the experiments. In a few instances the animals were not litter mates but were of approximately the same age and weight.

In one series of experiments immature rats were injected with the equivalent of 200 cc. of castrate urine (sufficient to produce luteinization at the end of 96 hours), and the animals were sacrificed at intervals of 48-96 hours. The ovaries were then examined in serial sections.

A similar experiment was performed using whole pregnancy urine.

In another series of experiments other animals were injected with 'Maturity Factor', 'A.P.L.', and castrate urine extracts. Laparotomies were performed at intervals varying from 26 to 72 hours after injections were started and one ovary removed. The animals were then sacrificed at different time intervals varying from 52 to 144

---

* Hiram N. Vineberg Research Fellow in Gynecology.

1 Frank, R. T., Salmon, U. J., and Friedman, R., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1666.

2 Salmon, U. J., and Frank, R. T., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 463.

† We are indebted to Ayerst, McKenna and Harrison of Montreal for a generous supply of the "Maturity Factor" and "A.P.L."

TABLE I.

Type of G.H. extract	Dosage, cc.	Rat No.	Laparotomy: Hr. after 1st inject.	Laparotomy: Ovarian reaction	Autopsy: Hr. after 1st inject.	Autopsy: Ovarian reaction
Castrate Urine	200	29			48	I
	200	30			72	I, III slight
	200	31			96	III, I "
Castrate Urine	100	1	60	I*	120	I, III "
	159	9	72	I strong	96	I, III
Castrate Urine	200	2	48	I, III* slight	96	III, I
	200	6	72	I strong	120	I, III
	200	24	48	I slight	96	III, I "
(M31UA42)‡	200	10	60	I "	120	I slight
	200	11	72	I "	120	I "
	200	12	96	I	144	III
Anterior Hypophyseal Extract ("Maturity Factor") (24 units/cc.)	2	1	30	I	52	III
	2	2	30	I slight	76	I slight, III
	2	5	38	I	98	III
	2	1A	48	I "	96	I, III slight
	2	7	48	0	120	0
	2	6A	50	I	120	III
	2	5A	64	I	144	III
	2	4	64	I, III slight	120	III
	2	2A	64	I, III "	144	III
	2	6B	72	I, III "	144	III
	2	3	72	I, III "	96	III, I
Whole Pregnancy Urine (P9U6)	3.5	18			48	0
	3.5	19			60	I, III slight
	2.5	20			72	I, III "
	3.5	21			96	III, I
Whole Pregnancy Urine (3402)	1	7			24	0
	1	8			48	I slight
	1	9			72	I, III slight
	1	23			96	III, I
A.P.L., 100 units/cc.	1	4	26	I	76	III, I
	1	10	32	0	82	0
	1	2	38	I slight	82	III slight I slight
	1	12	48	0	96	III very slight
	2	11	48	0	120	III, I slight
	2	7	49	I	98	I slight, III
	2	9	64	I and III	96	III slight
	2	10	72	III	96	III "

I* follicle stimulating effect. III* luteinizing effect.

hours after the first injection. The ovaries were serially sectioned. The results are presented in Table I.

It appears that in some cases the immature rat ovary will respond to gonadotropic hormone with a follicle stimulating reaction as early as 26 hours after the first injection. This reaction becomes progressively more marked and reaches its maximum at approximately 64-72 hours after injections are begun. After 64 hours luteinization begins to occur and progresses steadily thereafter. In the majority of animals luteinization reaches its maximum at the end of 96 hours. In one instance (M31UA42) only follicle stimulation was present at the end of 96 hours and 120 hours. At 144 hours, however, extensive luteinization was found.‡

From these experiments it appears that the ovaries of normal immature animals respond to gonadotropic hormone extracts when given in sufficient dosage, first by follicle stimulation which is followed, after a variable period of time, by luteinization. From this and the preceding study it is evident that at least 3 factors influence the production of luteinization, *viz.*, the method of extraction, the quantity of extract administered, and the time.

9029 C

Resistance of the Viruses of Poliomyelitis, Human Influenza and Swine Influenza to Intense Vibration.*

HENRY W. SCHERP AND LESLIE A. CHAMBERS. (Introduced by D. W. Bronk.)

From the Department of Pediatrics and the Johnson Foundation for Medical Physics, School of Medicine, University of Pennsylvania.

There is great need for a technique whereby the pathogenicity of viruses may be destroyed without altering the immunological properties of their antigenic constituents. The results of past work indicated that sonic vibration might be the agent by means of which this result could be accomplished. Thus, it was recently reported[1]

‡ From this it might appear that when a subthreshold dose of luteinizing factor is given, prolongation of the time beyond 96 hours may result in luteinization.

* This work was supported in part by grants from the President's Birthday Ball Commission for Infantile Paralysis Research, and from the Bureau of Animal Industry, U. S. Department of Agriculture.

1 Chambers, L. A., and Flosdorf, E. W., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 631.

that disintegration of *Bb. typhi* and *S. hemolyticus* by the action of sonic vibrations of audible frequency (9,000 c.p.s.) and high intensity resulted in the liberation, in an apparently unaltered state, of certain labile components of the antigenic complex of the organisms. Takahashi and Christensen[2] have reported that tobacco mosaic virus, in the form of the juice of infected leaves, was inactivated by exposure to sonic vibration of 450,000 c.p.s. frequency. Stanley[3] confirmed these results and observed in addition that a partially purified virus preparation was much less readily inactivated than was the crude leaf juice, and that in no case was inactivation possible when the irradiation was conducted *in vacuo* (absence of oxygen and cavitation). Hopwood,[4] on the other hand, found that vaccinia virus was not inactivated by exposure to supersonic vibration, but that there was indeed an increase in potency.

Accordingly, we have applied the sonic method to preparations of the viruses of poliomyelitis, human influenza and swine influenza, using the technique described for bacteria.[5] Under conditions of the experiments, the viruses proved to be completely resistant to the action of sonic vibration.

Poliomyelitis. The Philadelphia 1932 strain of the virus has been used in these experiments. Partially purified preparations for exposure to the sonic vibrations were made by submitting saline extracts of the spinal cords of monkeys (*Macaca mulatta*) which had succumbed to the experimental disease to a process of adsorption and elution similar to those described by Sabin[5] and Schaeffer and Brebner.[6] These methods and the properties of the products obtained by their use will be more fully described in another communication.[7] After the preparation had been exposed to sonic vibration, it was tested for the presence of active virus by intracerebral inoculation into normal monkeys, weighing not more than 5 pounds, under deep ether anesthesia. When the resulting infection was not in every respect clinically typical of poliomyelitis, the result was confirmed by histological study of the animal's spinal cord.†

So far as one is able to judge from the results of the inoculation

[2] Takahashi, W. N., and Christensen, R. J., *Science*, 1934, **79**, 415.

[3] Stanley, W. M., *Science*, 1934, **80**, 339.

[4] Hopwood, F. L., *Nature*, 1931, **128**, 748.

[5] Sabin, A. B., *J. Exp. Med.*, 1932, **36**, 307.

[6] Schaeffer, M., and Brebner, W. B., *Arch. Path.*, 1933, **15**, 221.

[7] Scherp, H. W., and Wolman, I. J., to be published.

† The authors are indebted to Dr. Irving J. Wolman for examining these specimens.

TABLE I.

Exposure of the Virus of Poliomyelitis to Sonic Vibration.

Oxygen was present unless otherwise noted. The temperature did not exceed 20° C at any time.

Exp. No.	Conc. virus exposed,* %	Length exposure, min.	Conc. tested in monkey, %	No. monkey	Incr. dose, cc.	Result of intracerebral test
1†	10	0	10	208	1	Complete paralysis, 10 days
		0	1	207	1.5	" " 6 "
		20	10	209	1.2	" " 12 "
		20	1	181	1	" " 10 "
2	10	0	10	220	1	" " 7 "
		5	10	218	1	" " 7 "
		15	10	219	1	" " 6 "
3	10	0	10	221	0.5	" " 8 "
		15	10	222	0.5	" " 7 "
4	10	0	10	249	1	" " 8 "
		60	10	248	1	" " 7 "
5	10	0	10	260	0.5	" " 6 "
				261	0.5	Part. paral., 9-10 d. Recovered
		60	10	258	0.5	Complete paralysis, 10 days
				259	0.5	" " 7 "
6	10	0	1.0	275	1	Temp. 105.4-106.4°F, 4-7 days‡
	crude	0	0.1	273	1	Complete paralysis, 10 days
	suspn.	30	1.0	274	1	" " 10 "
		30	0.1	272	1	Legs completely paralyzed, 12th day. Recovered partially

*Expressed in the same terms as the suspension from which the preparation was made. Thus, "10%" indicates that the eluate was equal in volume to the volume of the saline cord extract used in its preparation.

†Carried out in an atmosphere of hydrogen.

‡Histological examination of the spinal cord revealed no evidence of poliomyelitis.

test, which is inherently unsuitable for quantitative interpretation, the virus is entirely unaffected by even long exposure to intense sonic vibration. The results of several experiments are summarized in Table I. It is particularly noteworthy that, except for the first experiment which was conducted in an atmosphere of hydrogen, all tests were made in the presence of oxygen, under conditions favorable for the sonic activation of chemical systems.[8]

Buggs and Green have reported[9] that homogenization of herpetic rabbit brain in a colloid mill resulted in a suspension which produced herpes encephalitis in test rabbits with a much reduced incubation time and at much higher titer than that obtained by using material triturated in the customary manner in a mortar. Thus, using comparable doses, homogenized virus produced death in 12 to 24 hours, whereas triturated virus required 2 to 6 days. There was a direct relationship between the degree of dispersion of the infected tissue

[8] Flosdorf, E. W., Chambers, L. A., and Malisoff, W. M., *J. Am. Chem. Soc.*, 1936, **58**, 1069.

[9] Buggs, C. W., and Green, R. G., *J. Infect. Dis.*, 1936, **58**, 98.

and the activity of its virus content. The minimal fatal dose of homogenized virus was of the order of 0.0001 mg. of brain, or much less than is usually obtained with this virus. Unfortunately, the authors did not run a control series of their own with triturated material.

An experiment was carried out to determine if there was any evidence that a similar result could be obtained with poliomyelitis virus, using sonic vibration as the homogenizing agent. Accordingly, a spinal cord, freshly removed from a monkey completely paralyzed with poliomyelitis, was thoroughly ground in a mortar without abrasive, and made up to a 10% suspension in saline. The suspension was filtered through 2 layers of gauze to remove gross particles. Part of the filtrate was set aside for injection into monkeys without further treatment. The remainder was exposed to the action of the sonic apparatus for 30 minutes. The suspension was smoothly homogenized, and the insoluble matter settled out much more slowly than it did in the case of the untreated suspension. The results of the inoculation of these fractions into monkeys are shown as Experiment 6 in Table I. It was obvious that here was no result analogous to those obtained by Buggs and Green with herpes virus.

To check upon the relative efficiency of simple trituration and sonic homogenization in the extraction of soluble material from the nerve tissue, protein nitrogen and total Kjeldahl nitrogen determinations were carried out in duplicate upon the supernatant fluid ("saline extract") obtained when the suspensions were vigorously centrifuged. The sonic extract contained 0.51 mg. total nitrogen per cc. and 0.24 mg. protein nitrogen per cc. The corresponding figures for the extract prepared by simple trituration were 0.49 mg. and 0.29 mg. It would appear that sonic extraction liberated slightly more total solute but resulted in the denaturation of some fraction of the protein.[10]

Influenza. The strain of human influenza virus was the PR8; of swine influenza virus, the S15. These strains were originally obtained through the courtesy of Dr. Thomas Francis, Jr., and Dr. Richard E. Shope, of the Rockefeller Institute. Both strains were used for the experiments in the form of saline extracts of the lungs of mice that had succumbed to the experimental disease. The lungs were removed at autopsy with sterile precautions and stored in the frozen state at a temperature of −8 to −10°C. for not more than 2 weeks before use. In making the extract, the lungs were ground

[10] Chambers, L. A., and Flosdorf, E. W., *J. Biol. Chem.*, 1936, **114**, 75.

with powdered sterile pyrex glass in a mortar and made up to a suspension of the desired strength of 0.85% sodium chloride solution which was centrifuged at refrigerator temperature for 30 minutes at 2000 R.P.M. These extracts were cloudy with suspended material. Short exposure to sonic vibrations resulted in clarification but the cloudiness usually reappeared upon prolonged treatment. After exposure, the fractions were diluted in saline and portions of the dilutions were instilled intranasally into healthy albino mice 4 to 5 weeks old, under light ether anesthesia.‡ The mice were killed when moribund or at the end of the fifth to seventh day. Their lungs were then removed and examined for the lesions of influenzal pneumonia.

The results obtained with the 2 strains of influenza virus are

TABLE II.

Exposure of the Viruses of Human and Swine Influenza to Sonic Vibration. Oxygen was present in all cases. The temperature did not at any time exceed 20°C.

Exp. No.	Conc. virus exposed	Length exposure, min.	Mouse test dilutions.*			
			1:10	1:100	1:1,000	1:10,000
1	10%	0		A D5 D5	D5 2+ 2+	3+ 2+ 1+
	PR8	5		E D5 D5	4+ 2+ 1+	1+ 1+ 1+
		15		D3 D5 D5	D5 3+ 2+	2+ 1+ 1+
		30		E E D5	3+ 2+ 2+	2+ 1+ 1+
2	10%	0		D3 D3 2+	D5 D5 2+	A D5 3+
	PR8	5		D3 D4 D7	D5 3+ 2+	3+ 3+ 2+
		30		D4 D7	D5 3+	2+
3	10%	0		D6 D6 3+	2+ 2+ 2+	1+ 1+ 1+
	PR8	5		D6 D6 D6	2+ 2+ 1+	2+ 2+ OL
		30		D6 D6 3+	D6 2+ 1+	1+ 1+ 0
4	10%	0	2+ 3+ 3+	2+ 2+ 4+	1+ 1+ 0	A A 0
	S15 glycerolated	30	D5 D5 3+	1+ 1+ 2+	1+ 0 0	0 0 0
5	7.2%		1:1,400	1:14,000		
	PR8	0	D1 3+ 3+	D1 2+ 2+		
	Tissue sediment from above extract	30	E 3+ 3+	1+ 1+ 0		

*Dilutions are expressed in parts, wet weight, of lung tissue to parts of saline by volume.

Notation:

"A" Mouse died with atypical lung involvement.

"D3," "D4," etc. Mouse died with typical lesions of influenzal pneumonia in 3 days, 4 days, etc.

"E" Mouse eaten.

"L" Large, older Mouse—not as susceptible to the virus.

"0" No detectable influenzal lung lesions at autopsy.

"1+" Lesion of influenzal pneumonia involved up to ¼ of lungs at autopsy.

"2+" Lesion of influenzal pneumonia involved ¼ to ½ of lungs at autopsy.

"3+" Lesion of influenzal pneumonia involved ½ to ¾ of lungs at autopsy.

"4+" Lesion of influenzal pneumonia involved ¾ to all of lungs at autopsy.

‡ The authors take pleasure in acknowledging their indebtedness to Miss Dorothy R. Shaw, who carried out these animal tests.

summarized in Table II. The findings were entirely comparable to those obtained with the poliomyelitis virus. Even prolonged exposure to sonic vibration failed to affect the titer of the viruses, with the possible exception of the experiment in which glycerolated lungs were used as the source of the virus. In that case, the presence of glycerol was probably a factor of importance, since it has been shown that glycerol is one of many organic substances which are activated by intense sonic vibration in the presence of oxygen.[11]

An homogenization experiment (No. 5 of Table II) was carried out with the PR8 virus. The lungs were ground in a mortar without abrasive and made up in saline to a 7.2% suspension, which was then centrifuged for 10 minutes at 2000 R.P.M. The supernatant saline extract was set aside as a control and the sedimented tissue was suspended in the original volume of saline and homogenized by 30 minutes exposure to the sonic vibrations. It will be seen from the data in Table II that the virus content of the homogenized lung tissue suspension and of the saline extract were nearly the same, showing that simple trituration of the lung tissue resulted in only partial extraction of the virus. However, there was clearly no evidence of enhanced activity in the homogenized material.

Summary. The pathogenicity of preparations of the viruses of poliomyelitis, human influenza and swine influenza was not affected by exposure to intense sonic vibrations (9,000 c.p.s.) under conditions which suffice to disintegrate such bacteria as *Eb. typhi* and *S. hemolyticus*. No evidence was obtained that homogenization of virus-containing tissue by exposure to intense sonic vibration resulted in increased activity of the virus content.

[11] Chambers, L. A., unpublished data.

9030 P

Carboxymethylcysteine Metabolism, its Implications on Therapy in Cystinuria and on the Methionine-Cysteine Relationship.

Erwin Brand, Richard J. Block, Beatrice Kassell and George F. Cahill.

From the Department of Chemistry, N. Y. State Psychiatric Institute and Hospital and from the Squier Urological Clinic of the Presbyterian Hospital, New York.

In continuation of our experiments on sulfur metabolism, the behavior of carboxy-methyl-S-cysteine[1] (I) was investigated. (We are indebted to Dr. L. Michaelis for suggesting this compound for study.) Carboxyl-methyl-S-cysteine, like methyl-S-cysteine[2] does not support the growth of rats on a sulfur deficient diet, indicating that (I) does not yield cysteine (IV) in the course of its metabolism.

Three and six-tenths grams of (I) were administered to a normal human being, while 7.2 and 14.4 gm. respectively were given to 2 cystinuries. The substance was not toxic and yielded in the urine neither cystine nor SH compounds. In the normal, the sulfur of (I) was only partially oxidized (40%), the larger portion (60%) being excreted as undetermined neutral S. Part of this neutral S was apparently a disulfide, since there appeared in the urine a strong cyanide-nitroprusside reaction, while the Sullivan test remained negative. The urine was discarded before the probable nature of this disulfide was realized.

Following the ingestion of (I) by the cystinuric patients, about 15% of the extra sulfur excreted was inorganic sulfate and 85% undetermined neutral S. Cystine excretion remained practically unchanged as indicated by the Folin photometric method,[3] but when measured by the Sullivan and Lugg-Sullivan methods, it dropped from one gm. to 200 mg. per day.

These various findings seemed to indicate that part of (I) was excreted presumably as the unchanged compound, that part of it was oxidized to yield inorganic sulfate, and that another portion was excreted as an intermediate which contained sulfur in the form of a disulfide linkage. As a result of previous experiments,[4] and

[1] Michaelis, L., and Schubert, M. P., *J. Biol. Chem.*, 1934, **106**, 331.

[2] Block, R. J., and Jackson, R. W., *J. Biol. Chem.*, 1932, **97**, cvi; duVigneaud, V., Loring, H. S., and Craft, H. A., *J. Biol. Chem.*, 1934, **105**, 481.

[3] Kassell, B., *J. Biol. Chem.*, 1935, **109**, xlix.

[4] Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, 1935, **109**, 69; Brand, E., Cahill, G. F., and Block, R. J., *J. Biol. Chem.*, 1935, **110**, 399.

from the chemical formula of (I), it was reasonable to assume that the excretion of a disulfide resulted from the oxidation of an –SH compound derived from carboxymethylcysteine. The observations could therefore be explained by assuming that in the catabolism of (I), thioglycolic acid (II) is formed, which is excreted as dithiodiglycolic acid (III).

$$\underset{\text{Carboxymethylcysteine}}{\overset{\text{I}}{HOOC \cdot CH_2{-}S{-}CH_2 \cdot CH(NH_2) \cdot COOH}} \rightarrow \underset{\substack{\text{Thioglycolic}\\ \text{acid}}}{\overset{\text{II}}{HOOC \cdot CH_2 \cdot SH}} \rightarrow \underset{\substack{\text{Dithiodiglycolic}\\ \text{acid}}}{\overset{\text{III}}{(-SCH_2 \cdot COOH)_2}}$$

This conception seemed to be substantiated by the finding that both (II) and (III) markedly depress color formation in the Sullivan and Lugg-Sullivan reactions. Although (II) and (III) are readily soluble in ether, extraction of the cystinuric urines from the experimental periods did not yield any ether-soluble S-S compounds.

Our experiments[4] have indicated that the metabolic error in cystinuria is concerned with the handling of cysteine and that there are probably present in the kidney of the cystinuric individual abnormally large amounts of cysteine. It was therefore conceivable that in the presence of large amounts of cysteine (IV) the thioglycolic acid (II) resulting from the catabolism of (I) was excreted as a mixed disulfide such as (V).

$$\underset{\text{Thioglycolic acid}}{\overset{\text{II}}{HOOC \cdot CH_2 \cdot SH}} + \underset{\text{Cysteine}}{\overset{\text{IV}}{HS \cdot CH_2 \cdot CH(NH_2) \cdot COOH}} \rightarrow \underset{\text{Mixed disulfide}}{\overset{\text{V}}{HOOC \cdot CH_2 \cdot S{-}S \cdot CH_2 \cdot CH(NH_2) \cdot COOH}}$$

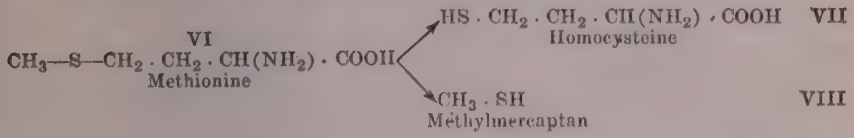
Although a compound such as (V) has as yet not been isolated, its presence is indicated by the following observations. On reduction (Zn + HCl) of 25-100 cc. of the experimental urine it was possible to detect the characteristic odor of thioglycolic acid. Ether extracts of the reduced urine contained a substance with acidic properties, which gave a strong nitroprusside reaction, Goddard and Michaelis's[5] test for thioglycolic acid and a reaction curve in the Folin-photometric determination similar to that given by thioglycolic acid.

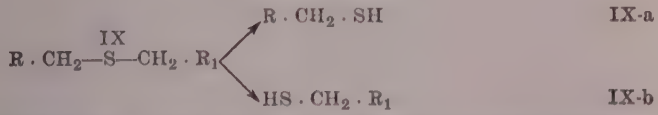
Mixed disulfides are quite soluble. The probable formation of a compound such as (V) following the administration of (I) to cystinuric patients may, therefore, have therapeutical implications regarding the formation of cystine stones. In this connection it is interesting to note that during the period of feeding of (I) cystine crystals disappeared from the urine of one of the cystinuric patients.

Since methionine (VI) can be split[7] to yield homocysteine (VII)

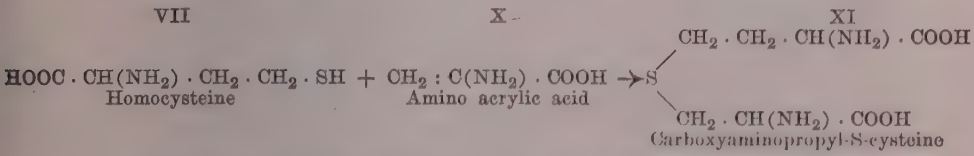
[5] Goddard, D. R., and Michaelis, L., *J. Biol. Chem.*, 1934, **106**, 605.

or methylmercaptan (VIII) and carboxymethylcysteine to yield thioglycolic acid (II), it appears that compounds of the general formula (IX) may be split to yield (IX-a) or (IX-b), depending upon conditions and the nature of the substituents R and R_1.

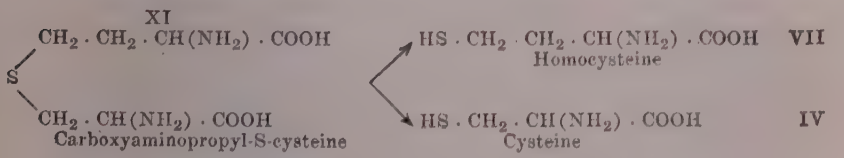




It has been stated[4] that, along the line suggested by model experiments,[6] the conversion of homocysteine (VII) into cysteine (IV) may be accomplished by a reaction of (VII) with amino acrylic acid (X) (or with its peptides) to yield carboxyaminopropyl-S-cysteine (XI) (or its peptides).



It is therefore conceivable that (XI) may be split to yield cysteine (IV).



These conceptions form the basis for a working hypothesis regarding some of the mechanisms by which the conversion of methionine into cysteine may be accomplished in intermediary metabolism, and also indicate the importance of synthesizing (XI) and its derivatives for chemical and metabolic investigations. (XI) has on one occasion been isolated from wool by Kuester and Irion.[8]

Note. Recently, 14.4 gm. of (I) were administered to another human being. The urine of the experimental period contained approximately 800 mg. of the mixed disulfide (V).

[6] Nicolet, B. H., *Science*, 1935, **81**, 181; *J. Biol. Chem.*, 1932, **95**, 389; Bergmann, M., *Naturwissinschaften*, 1934, **22**, 135.

[7] Butz, L. W., and duVigneaud, V., *J. Biol. Chem.*, 1932-33, **99**, 135; *cf.* Virtue, R. W., and Lewis, H. B., *J. Biol. Chem.*, 1934, **104**, 59.

[8] Kuester, W., and Irion, W., *Z. physiol. Chem.*, 1929, **184**, 225.

9031 C

Effect of Dinitrophenol and Dinitrocresol on Oxygen Consumption of Diapause and Developing Embryos.*

J. H. Bodine and E. J. Boell.

From the Zoological Laboratory, State University of Iowa.

The stimulating action of the dinitrophenols on the oxygen consumption of cells and tissues is generally thought to reside in an acceleration of metabolic processes involving primarily the oxidation of carbohydrate. Dodds and Greville[1] showed that the extra oxygen uptake induced by 4,6-dinitro-o-cresol (DNC) in kidney slices had a R.Q. of 1. Ehrenfest and Ronzoni[2] demonstrated an increased oxidation of carbohydrate in yeast treated with dinitrophenol (DNP). Later[3] they demonstrated that DNP was without effect in iodoacetate poisoned muscle. Clowes and Krahl[4] showed that the R.Q. of sea urchin eggs in the presence of DNC remained at the level characteristic of untreated eggs, *viz.*, 0.93 to 0.95. Field and Tainter[5] present data to show that the stimulation of respiration in yeast by DNC is maximal in the presence of glucose.

In view of the fact that most of the work dealing with the effects of dinitrophenols has been done on biological systems whose normal fuel is in the main carbohydrate, it seems advisable to investigate the effects of DNP and DNC† on the oxidative metabolism of the grasshopper embryo (*Melanoplus differentialis*) where metabolism is predominantly at the fat level.[6] This paper will present preliminary facts relating to the stimulating effects of DNP and DNC on respiration together with a description of the influence of carbon monoxide and cyanide on the increased oxygen uptake.

The method of preparing the embryos for experiments has been described previously.[7] O_2 uptake was measured in the Barcroft-

* Aided by a grant from the Rockefeller Foundation for Research in Cellular Physiology.

1 Dodds, E. C., and Greville, G. D., *Nature*, 1933, **132**, 966.

2 Ehrenfest, E., and Ronzoni, E., Proc. Soc. Exp. Biol. and Med., 1933, **31**, 318.

3 Ronzoni, E., and Ehrenfest, E., *J. Biol. Chem.*, 1936, **115**, 749.

4 Clowes, G. H. A., and Krahl, M. E., *Publ. Eli Lilly Research Lab.*, 1934.

5 Field, J., 2nd, and Tainter, E. G., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 168.

† The compounds used were 2,4-dinitrophenol and 3,5 dinitro-o-cresol (Eastman Kodak Company).

6 Boell, E. J., *J. Cell and Comp. Physiol.*, 1935, **6**, 369; Bodine, J. H., and Boell, E. J., unpublished data, 1936.

7 Bodine, J. H., and Boell, E. J., *J. Cell. and Comp. Physiol.*, 1936, **8**, 357.

Warburg apparatus for a period of from 1 to 3 hours in order to obtain a normal rate of oxygen consumption. DNP was then added to the manometer contents from the side bulb and the effect of the addition noted for a second period of from 1 to 3 hours. In the experiments involving the use of cyanide the technique of van Heyningen[8] was used.

The comparative stimulating effects of various concentrations of DNP on the oxygen uptake of diapause (blocked) and developing embryos is indicated in Table I. The Q_{O_2} values represented

TABLE I.

Oxygen Uptake of Diapause and Developing Embryos in Presence of Dinitrophenol. The embryos were suspended in a medium consisting of 0.9% NaCl, 0.02% KCl, 0.02% $CaCl_2$, M/100 phosphate buffer to give pH 6.2, and dinitrophenol to make concentration indicated.

Q_{O_2} = mm.^{3} O_2/hour/mg. (dry weight) of embryo.

Molar conc. DNP × 10^{-5}	Diapause Q_{O_2}	Diapause %	Developing Q_{O_2}	Developing %
0 (control)	.75	100	1.55	100
.125	.90	120	1.71	110
.25	1.46	195	2.23	144
.5	1.88	250	3.43	221
1.	1.93	258	3.67	237
2.5	2.55	340	4.28	276
3.	2.56	342	—	—
5.	2.16	288	3.87	250
25.	.85	113	—	—

for each type of embryo are averages of from 5 to 25 experiments in each concentration group. Maximum stimulation both in diapause and developing embryos is achieved with a concentration of DNP of 2.5×10^{-5} molar. Although the percentage increase in respiration is higher in diapause than in developing embryos, the absolute value of oxygen consumption is greater in embryos of the latter type since normally they respire at a rate 2 to 3 times that of blocked embryos.

DNC is more than twice as active physiologically as DNP for the concentration of this substance which produces maximal stimu-

TABLE II.

Effect of Carbon Monoxide on Stimulation of Respiration Produced by Dinitrophenol.

Concentration DNP = 2.5×10^{-5} molar; CO/O_2 = 95/5.

	Diapause embryos Q_{O_2}	Diapause embryos %	Developing embryos Q_{O_2}	Developing embryos %
Control	.73	100	1.48	100
CO	.70	96	.55	37
DNP	2.01	276	3.39	229
DNP + CO	1.00	137	.42	28

[8] Heyningen, W. E. van, *Bioch. J.*, 1935, **29**, 2036.

lation is 1×10^{-5} molar. With both DNP and DNC the increased rate of respiration is maintained for several hours without undergoing appreciable change.

Table II shows that the stimulating effect of DNP is completely restricted by carbon monoxide in the case of developing embryos. With diapause embryos CO normally has only a slightly depressing effect; moreover, it does not suppress completely the extra oxygen uptake induced by DNP (compare DeMeio and Barron[9]).

Cyanide (Table III) when added to embryos, either before, after or simultaneously with the DNP, can reduce stimulation, prevent it entirely or depress respiration below the normal level. With developing embryos there seems to be no antagonism between DNP and the stronger concentrations of cyanide.

TABLE III

Stimulation of Respiration by Dinitrophenol in Presence of Cyanide. Concentration of DNP $= 2.5 \times 10^{-5}$ molar.

Molar KCN $\times 10^{-5}$	Q_{O_2} Diapause embryos				Q_{O_2} Developing embryos			
	KCN	%	KCN + DNP	%	KCN	%	KCN + DNP	%
0 (Control)	.66	100	2.35	356	1.47	100	3.02	206
5	.70	106	1.56	236	1.46	99	2.62	178
50	.56	85	1.16	176	1.05	71	.96	65
100	.29	44	.60	91	.48	33	.55	37

A slight antagonism between DNP and KCN seems to be apparent with diapause embryos.

That the stimulation of respiration in both types of embryos by DNP or DNC involves the functioning of the normal oxidative mechanism of the cell is suggested by the sensitivity of the stimulation to carbon monoxide and cyanide, and also, by the fact that the temperature coefficients of the normal and DNP stimulated respiration are approximately the same. The data suggest, moreover, that the dinitrophenols can increase respiration in biological systems where carbohydrate metabolism does not predominate.

[9] DeMeio, R. H., and Barron, E. S. G., PROC. SOC. EXP. BIOL. AND MED., 1934, 32, 36.

9032 C

Inositol and the Respiration of Brain.

LESLIE YOUNG.* (Introduced by P. A. Shaffer.)

From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.

By means of manometric measurements using the Warburg apparatus Das and Guha[1] claim to have shown that the oxygen consumption of various rat tissues is greater when inositol is present in the suspension medium than in control experiments in which it is absent. This effect was found in the case of brain, heart, kidney and liver tissue, being most marked in the case of brain tissue. After experiments of 2-3 hours' duration Das and Guha found that when inositol was present unwashed brain tissue showed an oxygen consumption about 35% greater than that of the control (one experiment reported) and in the case of washed brain tissue (3 experiments reported) increases up to about 90% were found.

Since it has not been possible to ascribe any biochemical significance to the considerable amounts of inositol shown to be present in brain (Thudichum,[2] Young[3]) the following work was undertaken to confirm and extend the above findings of Das and Guha.

The experimental procedure adopted in the case of the results recorded in Series I and II was that used by Das and Guha. White rats were used, and after decapitation the brains were removed as rapidly as possible, sliced, and the slices cut into small pieces. The tissue was transferred to Warburg flasks containing 1 ml. of Ringer-Locke solution, 1 ml. of Na_2HPO_4-NaH_2PO_4 buffer (pH 7.4) and either 0.5 ml. of water or 0.5 ml. of 0.1 M inositol solution. The CO_2 was absorbed with 0.2 ml. of 20% KOH solution in the inset of the flask, and the temperature of the thermostat was 37.3°C. In experiments in which washed brain was used, the finely cut tissue was washed twice in a centrifuge tube with Ringer-Locke solution as described by Das and Guha. Washing the finely cut brain several times was not found to affect the results greatly.

It must be recognized that with the above technique certain factors are involved which can introduce variations in replicate experiments. When the Warburg flasks contain air the brain tissue does not re-

* Commonwealth Fund Fellow in Biochemistry.

1 Das, N., and Guha, B. C., *Z. physiol. chem.*, 1935, **231**, 157.

2 Thudichum, J. L. W., *Die chemische Konstitution des Gehirns des Menschen und der Tiere*, Tübingen, 1901.

3 Young, L., *Biochem. J.*, 1934, **28**, 1435.

ceive as much oxygen as it can use. This is demonstrated in Series III in which parallel experiments to those in Series II were performed using oxygen instead of air. Furthermore with low oxygen tensions variations in the state of division of the tissue in different flasks tend to cause larger variations in the oxygen consumption than when pure oxygen is used. Since the oxygen uptake of cerebral cortex is greater than that of white matter, experiments in which whole brain is used are open to variation due to varying proportions of grey and white matter in different flasks. In view of these facts it is desirable that conclusions be drawn only from the mean results of a sufficient number of experiments to eliminate these possible variations.

The results given in Series I and II show that when this was done it was not possible to confirm the findings of Das and Guha on rat brain. Furthermore no inositol effect was found in the case of similar experiments with washed whole brain in oxygen (Series III) or with unwashed cerebral cortex in air. In the control experiments of Das and Guha there was a rapid falling off in the rate of oxygen consumption which has not been found in the present work. The most marked effect with added inositol was obtained by Das and Guha in those experiments in which the rate of oxygen uptake of the controls fell off to the greatest extent. The writer has not found either in this or other work that the respiration rate of brain tissue in the presence of glucose decreases to any marked degree over periods of 3 hours, and the control experiments for rabbit cerebral cortex reported in Series IV indicate little change in rate even over a period of 5 hours.

Other experiments were performed in which the more satisfactory phosphate medium described by Dickens and Greville[4] replaced the Ringer-Locke-phosphate medium used in the above experiments. Furthermore since it is desirable that the smallest possible changes should be made in the concentration of the suspension medium 0.2 ml. of 0.25 M inositol solution (or water) was added instead of the 0.5 ml. of 0.1 M inositol solution (or water) added in the above experiments. Under these conditions the oxygen uptakes in control experiments with either rat cerebral cortex or rat whole brain in either air or oxygen did not show any significant differences from those found in experiments when inositol was present. A similar result was obtained (See Series IV) in a series of experiments with rabbit cerebral cortex in which tissue slices were allowed to respire for 5 hours in oxygen and in which 0.2 ml. of 0.9% NaCl solution was added to the controls instead of 0.2 ml. of water.

[4] Dickens, F., and Greville, G. D., *Biochem. J.*, 1935, **29**, 1468.

TABLE I.

Series	No. of animals used	No. of pairs of exp.	Hr.	Mean oxygen uptake in cmm. per mg. (dry wt.) of tissue: Controls	Inositol added
I. Unwashed whole brain (rat) in air	4	5	1	5.2	5.1
			2	5.1	5.1
			3	4.7	4.7
			Total	15.0	14.9
II. Washed whole brain (rat) in air	3	6	1	5.3	5.6
			2	5.1	5.5
			3	4.7	5.1
			Total	15.1	16.2
III. Washed whole brain (rat) in oxygen	3	4	1	10.3	9.9
			2	10.0	10.1
			3	8.2	8.4
			Total	28.5	28.4
IV. Unwashed cerebral cortex (rabbit) in oxygen	1	5	1	13.9	13.7
			2	14.5	14.6
			3	14.2	14.4
			4	13.5	13.9
			5	13.2	13.7
			Total	69.3	70.3

It seems likely that even if inositol is involved in brain respiration, the above experiments might give no evidence of this. Inositol is not easily extracted from brain and it is possible that there is sufficient inositol present in brain, even after washing, to supply the needs of the tissue over the period of the experiments. Quastel and Wheatley[5] described a technique by which the effect of a substrate on the brain was studied by adding it after the tissue had been allowed to respire for some time in the absence of any added substance capable of being oxidized by the brain. Under such conditions the brain became depleted of its own oxidisable material before the addition of the substrate under investigation. This procedure was used in the present work. Rat whole brain was allowed to respire in oxygen in Dickens-Greville phosphate medium (2.0 ml.) from which glucose was absent. After a period of depletion the following solutions were added to the tissue from the side arms of the Warburg flasks in 4 parallel experiments: (1) 0.2 ml. water, (2) 0.2 ml. 0.1 M glucose solution, (3) 0.2 ml. 0.1 M inositol solution, (4) 0.1 ml. 0.2 M glucose solution and 0.1 ml. 0.2 M inositol solution. An example of the results obtained in such experiments is

[5] Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1932, **26**, 725.

shown graphically in Fig. 1. It is seen that the oxygen consumption of the tissue to which inositol was added continued to fall off at the same rate as the control without substrate. Addition of glucose alone caused some increase in the rate of oxygen consumption with subsequent stabilization at this level. When inositol was added at the same time as glucose no greater effect was detectable than in the case of glucose alone. A negative result was also obtained in a similar experiment in which rabbit cerebral cortex was used.

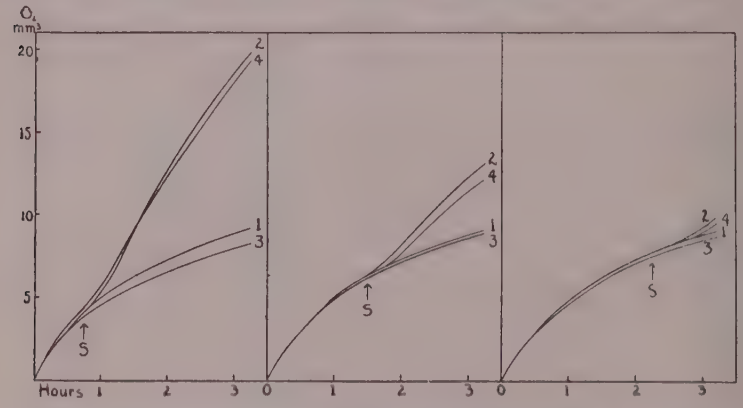


FIG. 1.

Graphs showing the effect on oxygen consumption obtained on adding (1) water, (2) glucose solution, (3) inositol solution, (4) glucose + inositol solution, to rat whole brain which had been allowed to respire without added substrate for varying periods. The additions were made at the points marked S. The oxygen consumption is expressed as cmm. of oxygen per mg. (dry weight) of tissue.

Even if inositol does play a part in the aerobic processes occurring in the brain, experiments of the type described above might well fail to give evidence of this. The purpose of this communication is merely to show that it has not been possible to confirm the findings of Das and Guha with regard to brain tissue and to report experiments under other conditions which have likewise failed to indicate that inositol has any significant effect on the respiration of brain.

9033 C

Calcium-Protein Relation in Hyperproteinemia: Total and Diffusible Serum Calcium in Lymphogranuloma Inguinale and Myeloma.

ALEXANDER B. GUTMAN AND ETHEL BENEDICT GUTMAN.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

A direct proportionality between calcium and protein concentrations has been demonstrated in sera of less than normal protein content where there was no disturbance of calcium metabolism.[1] This relation could be expressed by an empirical regression equation:[2, 3]

I. $\text{Total Ca} = m \cdot \text{total protein} + b$ (where m and b are constants).

It follows from equation I—and it is generally inferred, provided hyperphosphatemia is not present—that elevated calcium values are to be expected in sera of *high* protein content. This plausible inference would seem to be justified by the established fact that a significant proportion, approximately half, of the total calcium in normal serum is bound by protein. The inference appears to be supported by the co-existence of hyperproteinemia and hypercalcemia in cases of multiple myeloma.

However, in lymphogranuloma inguinale, in which the serum protein content may exceed 10%, hypercalcemia is not observed[4, 5] (Table I); nor do we find hypercalcemia accompanying hyperproteinemia encountered occasionally in hepatic cirrhosis, lymphosarcoma, tuberculosis, etc.[5] In multiple myeloma, moreover, the association of hypercalcemia with hyperproteinemia is erratic.[6, 7] Of 57 published cases of multiple myeloma in which both protein and calcium were determined (including 14 of our own), hyperproteinemia occurred in 35; of which number, hypercalcemia was present in 23. In 18 cases of multiple myeloma, hypercalcemia was associated with

1 Salvesen, H. A., and Linder, G. C., *J. Biol. Chem.*, 1923, **58**, 617.

2 Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1927, **71**, 723.

3 McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.

4 Williams, R. D., and Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 91.

5 Gutman, A. B., Gutman, E. B., Jillson, R., and Williams, R. D., *J. Clin. Invest.*, 1936, **15**, 475.

6 Robbins, C. L., and Kydd, D. M., *J. Clin. Invest.*, 1935, **14**, 220.

7 Cantarow, A., *Am. J. Med. Sci.*, 1935, **189**, 425.

normal or low serum protein levels. In several instances, the serum calcium remained unchanged or fell as the serum protein content rose; or falling protein levels were accompanied by unchanged or higher calcium levels.

Contrary to prevailing opinion, the direct proportionality between calcium and total protein in sera of low or normal protein content does not obtain in sera with increased protein content (Fig. 1).

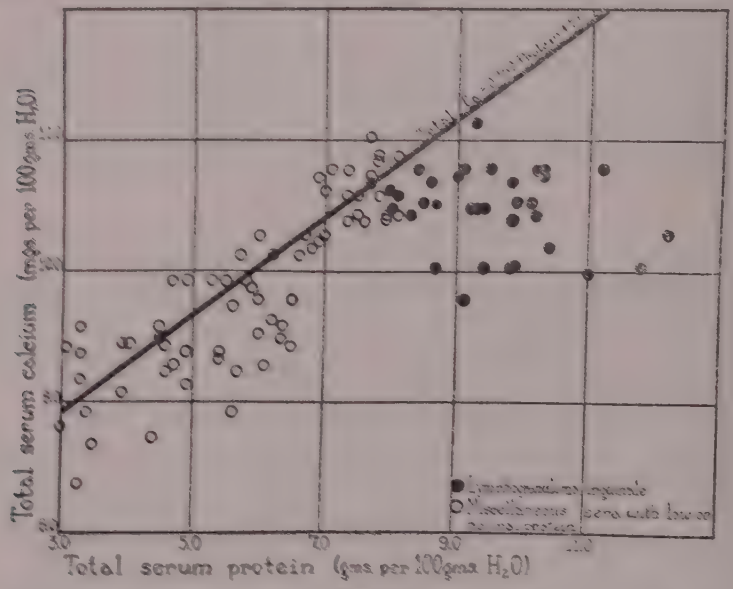


FIG. 1.

Our results on sera with normal protein, and protein low as a result of renal disease or malnutrition, show a direct proportionality between calcium and total protein which is in fair agreement with the mean empirical equation: Total Ca = 0.752 Protein + 5.6, given by McLean and Hastings.[4] This relation does not obtain in lymphogranuloma inguinale with hyperproteinemia (for values see [5]). Only cases with serum inorganic P of 2.5-5.0 mg. % have been used throughout.

Apart from some cases of multiple myeloma, where bone destruction co-exists, the total serum calcium does not rise in hyperproteinemia, but is maintained at normal levels.

The discrepant relation of calcium to protein in our cases of lymphogranuloma inguinale is not due to hyperphosphatemia, which was not present (Table I); or to a decrease in calcium not bound to protein, since the diffusible calcium fraction showed no convincing fall (Table I) and our patients show no evidence of tetany. The discrepancy results chiefly because despite the rise in total serum protein, there is no appreciable increase in the protein-bound calcium fraction.

TABLE I.
Total Protein and Calcium Fractions of the Serum in 5 Cases of Lymphogranuloma Inguinale (L.I.) and 3 Cases of Multiple Myeloma (M.M.).

		Serum							
							Diffusible Ca		
No.	Diagnosis	Total protein %	Alb. %	Glob. %	Euglob. %	Total Ca mg. %	mg. %	% of total Ca	Inorg. P mg. %
1.	L.I.	11.1	3.3	7.8	3.2	9.6	5.7	59	3.6
2.	L.I.	9.6	3.7	5.9	2.3	9.5	4.9	52	3.6
3.	L.I.	9.0	3.5	5.5	2.3	9.3	6.3	68	3.3
4.	L.I.	9.0	3.8	5.2	1.4	9.9	6.6	66	3.0
5.	L.I.	8.1	3.3	4.8	1.5	9.4	4.9	52	3.3
6.	M.M.	9.9	2.9	7.0	....	16.7	8.2	49	4.8
7.	M.M.	9.5	2.4	7.1	5.5	8.2	5.4	66	3.9
8.	M.M.	6.1	....	....	....	16.0	9.0	56	3.0

Mean % diffusible Ca/total Ca in normal sera = 61%.
No correction has been made either in normal or pathologic sera for Ca taken up by the collodion membranes.

The protein-bound calcium fraction fails to increase in hyperproteinemia due to lymphogranuloma inguinale for at least 2 reasons: 1. There is often some decrease in calcium bound to albumin since many of our cases show low normal or low albumin levels,[5] (Table I). Precisely how much lowering of the protein-bound calcium fraction is so effected cannot be determined because the calcium bound per gram albumin under the conditions obtaining in these sera is not known. Such approximations as can be made indicate that the fall in albumin in our cases is insufficient to account for the absence of definite hypercalcemia. 2. There remains the alternative that the protein-bound calcium fraction does not rise because a part (or all) of the globulin fraction does not bind a significant amount of calcium under the conditions existing in these sera. We suggest that this is the most important cause of the discrepant relation of calcium to protein in hyperproteinemia due to lymphogranuloma inguinale.*

* The discrepancy is a consequence of the fact that whereas *hypo*proteinemia is chiefly the result of loss of *albumin*, which binds an appreciable amount of calcium, *hyper*proteinemia is due to an increase in the *globulin* fraction, which is generally believed to bind significantly less calcium under the conditions obtaining in serum. Equation I in its present form does not allow for this difference. Expanding the term "m · protein" to "m_1 · albumin + m_2 · globulin" does not wholly resolve the difficulty, if, as is generally believed, m_2 is significant though small, since this would imply some increase in serum calcium with marked hyperglobulinemia. Our data on lymphogranuloma inguinale, however, reveal no upward trend whatever in total serum calcium levels as the serum globulin content increases (Fig. 1); *i. e.*, no significant amount of calcium is bound under these conditions either by the serum globulin fraction *in toto*, as Bendien and Snapper suggest[8], or by that

In multiple myeloma, as is well known, an absolute increase in the protein-bound calcium fraction may occur (Table I), but apparently only in cases presenting hypercalcemia, and then irrespective of whether or not the serum protein content is increased (Table I). It is suggested that an increase in protein-bound calcium in multiple myeloma with hyperproteinemia is a consequence not of the presence of increased globulin in the blood, but of dissolution of bone due to skeletal destruction. That the majority of cases of multiple myeloma presenting hyperproteinemia also exhibit hypercalcemia may mean only that myelomatosis severe enough to cause hyperproteinemia is likely to be extensive enough to produce widespread skeletal damage, with resulting hypercalcemia.

Conclusions. 1. Contrary to the prevailing view, hyperproteinemia (hyperglobulinemia) is not "a cause of" or "responsible for" hypercalcemia; it does not lead to abstraction of calcium from the bones either directly, or indirectly by upsetting the mass law relation[8] in the blood. In hyperproteinemia, even in the absence of hyperphosphatemia, there is no proportionality between total serum protein and serum calcium levels, and no inference as to the calcium content of the blood can be drawn from the total protein content.

2. Where hypercalcemia does occur in conjunction with hyperproteinemia, as in some cases of multiple myeloma, the calcium increase may well be due, not to hyperproteinemia, but to the complication of co-existent bone destruction by neoplastic tissue; like the hypercalcemia occurring occasionally with metastatic osteolytic carcinoma, in which serum protein levels are normal or low.[6, 9] The influx of Ca^{++} caused by bone destruction leads to an increase in protein-bound as well as in ionized calcium, with reëstablishment at higher levels of an equilibrium between these 2 fractions predictable by mass law considerations.[10] The absolute increase in the protein-bound calcium fraction, therefore, appears to be a *result* and not a cause of the hypercalcemia; the ratio diffusible Ca:total Ca remaining reasonably constant whether the serum protein is increased (multiple myeloma) or normal (hyperparathyroidism).

---

part of the globulin fraction composing the increase in serum protein content (usually chiefly euglobulin).

In this study, only the diffusible calcium fraction was determined, no attempt being made to estimate the calcium ion concentration either directly or from mass-law considerations.[8]

[8] Bendien, W. M., and Snapper, I., *Biochem. Z.*, 1933, **260**, 105.

[9] Gutman, A. B., Tyson, T. L., and Gutman, E. B., *Arch. Int. Med.*, 1936, **57**, 379.

[10] McLean, F. C., Barnes, B. O., and Hastings, A. B., *Am. J. Physiol.*, 1935, **113**, 141.

3. An increased protein content of the serum is not essential to hold in solution such high calcium concentrations as occur in disease. Loeb and Nichols[11] showed that the calcium bound per gram protein is a function of the calcium concentration, the protein normally present in serum binding more calcium at increased than at normal calcium levels. This occurs, presumably, in hyperparathyroidism. In multiple myeloma with both hypercalcemia and hyperproteinemia, it seems not unlikely that most of the increase in calcium bound to protein is calcium bound by *albumin;* and little, if any, calcium is bound by the euglobulin increment responsible for the hyperproteinemia.

4. The discrepant relation of protein to calcium in hyperproteinemia is only a special case of the discrepant relation of protein to total base in hyperproteinemia. As was pointed out elsewhere,[5] the assumption that all of the serum globulin fraction in hyperglobulinemia binds as much base as the factor '*B* globulin' in general use[12] calls for, leads to the result that the sum of total determined acids appears to exceed the total base.

Methods. Serum calcium, inorganic phosphorus, total proteins and protein fractions were determined by methods described elsewhere.[5] The diffusible calcium fraction was estimated by ultrafiltration of 10-15 cc. serum samples through collodion sacs in a Simms apparatus[13]; positive pressures of 40-50 mm. Hg. were applied, the serum being maintained under normal CO_2 tensions. We are indebted to Dr. H. S. Simms and Mr. A. Stolman for their help.

---

11 Loeb, R. F., and Nichols, E. G., *J. Biol. Chem.*, 1927, **72**, 687.

12 Van Slyke, D. D., Hastings, A. B., Hiller, A., and Sendroy, J., Jr., *J. Biol. Chem.*, 1928, **79**, 769.

13 Simms, H. S., *J. Gen. Physiol.*, in press.